Diversity of vaginal microbiota associated with bacterial vaginosis and the impact on pregnancy outcomes in HIV-infected and uninfected women.

By

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List of abbreviations

AF	Amniotic fluid
ANC	Antenatal clinic
ANOVA	Analysis of Variance
ART	Antiretroviral treatment
BA	Blood agar
BIOM	Biological observation matrix
BLAST	Basic local alignment search tool
BV	Bacterial vaginosis
BVAB	Bacterial vaginosis associated bacterium
CAMERA	Community Cyberinfrastructure for Advanced Marine Microbial Ecology Research and Analysis
CD4	Cluster of Differentiation 4
CDC	Cholesterol-dependent cytolysin
CRISPRs	Clustered regularly interspaced short palindromic repeats
CST	Community state type
ELISA	Enzyme-linked immunosorbent assay
FGF basic	Growth factor-basic
FGT	Female genital tract
G-CSF	Granulocyte colony-stimulating factor
GBS	Group B streptococcus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GV	Gardnerella vaginalis
H_2O_2	Hydrogen peroxide
HAART	Highly active antiretroviral treatment
HIV	Human Immunodeficiency virus
HSV	Herpes simplex virus
HUMAnN	A pipeline determining the presence/absence and abundance of microbial pathways in a community from metagenomic data
IFN-γ	Interferon gamma
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
IL-1β	Interleukin 1 beta
IL-2	Interleukin-2
IMG	Integrated microbial genomes

IP-10	Interferon gamma-induced protein (CXCL10)
IUGR	Intrauterine growth restriction
LBW	Low birth weight
LEfSe	Linear discriminant analysis effect size
MAC	MacConkey
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1 (MCP-1/CCL2)
MEGAN	Metagenome analyse
MG-RAST	Metagenomics Rapid Annotation using Subsystem Technology
MIP-1α	Macrophage inflammatory protein 1-alpha (Chemokine (C- C motif)ligand 3 (CCL3)
MIPip-1β	Macrophage inflammatory protein -1β (CCL4)
MiSeq	An integrated instrument that performs clonal amplification, genomic DNA sequencing, and data analysis with base calling, alignment, variant calling, and reporting in a single run.
MRCZ	Medical Research Council of Zimbabwe
MUSCLE	Multiple Sequence Comparison by Log- Expectation
NGS	Next generation sequencing
NMDS	Non-metric multidimensional scaling
OECD	Organisation for Economic Co-operation and Development
ORF	Open reading frames
OTU	Operational taxonomic units
PBS	Phosphate buffered saline
PCoA	Principal coordinate analysis
PDGF-BB	Platelet-derived growth factor (PDGF)
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
POC	Point of care (test)
PROM	Premature rupture of membrane
PRR	Pathogen recognition receptors
PTB	Preterm birth
PTD	Preterm delivery
PyNAST	Python nearest alignment space termination
QIIME	Quantitative Insights into Microbial Ecology.
RANTES	Regulated on Activation, Normal T Expressed and Secreted(CCL5)

RCZ	Research Council of Zimbabwe
RDP	Ribosomal Data Base project
RNA	Ribonucleic acid
RPL	Recurrent pregnancy loss
SCFAs	Short chain fatty acids
SDS	Sodium dodecyl sulfate
SGA	Small for gestational age
SIGLECS	Sialic acid binding immunoglobulin like lectins
SILVA	Latin silva, forestrRNA
SLPI	Secretory leukocyte peptidase inhibitor
ST	Serotype
STAMP	Statistical Analysis of Metagenomic Profiles
STI	Sexually transmitted diseases Infections
sTNFR1.	Soluble tumour necrosis factor receptor-1
Th	T-helper
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
UCLUST	An algorithm used to divide a set of sequences into clusters.
UCT	University of Cape Town
UN	United Nations
UPARSE	Algorithm for generating clusters (OTUS) from next-generation sequencing reads of marker genes such as 16S rRNA
UTI	Urinary tract infections
UZ	University of Zimbabwe
VAMPS	Visualization and analysis of microbial population structures.
VEC	Vaginal epithelial cells
VEGF	Vascular endothelial growth factor
WHO	World health organisation

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Abstract

The composition of vaginal microbiota in pregnancy is important as it may influence susceptibility to adverse pregnancy outcomes. Dysbiosis of the vaginal microbiota caused by the replacement of protective resident microorganisms such as *Lactobacillus spp*. by anaerobic bacteria is known as bacterial vaginosis (BV). BV is prevalent in women of African descent and may be a cause of higher rates of adverse birth outcomes in these women. Adverse birth outcomes have been reported to be higher in HIV-infected women both on treatment or treatment naïve compared to HIV-uninfected women. The relationship between the vaginal microbiome, HIV and pregnancy outcomes has not yet been established in Zimbabwean women, nor reported in sub Saharan Africa, where the burden of disease is high. Identification of specific organisms and immune factors associated with adverse pregnancy outcomes could lead to interventions or diagnostic algorithms to prevent and predict these outcomes in this population.

We recruited 420 pregnant Zimbabwean women [48 (11.4%) HIV infected, 372 (88.6%) HIV uninfected], between 13-35 weeks of gestation with a median gestational age of 30 weeks. Vaginal swabs were collected at enrollment and women were followed until pregnancy outcome was determined. Bacterial DNA was extracted from the vaginal swabs using an optimized Phenol chloroform extraction method with an addition of an enzymatic cocktail step. Library preparation was done using the Universal primers (515F/806R) for the hypervariable V4 region of the 16S rRNA gene. For the first PCR KAPA Hotstart + primers (Roche Lifescience, UK), were used for amplification. After amplification AMPure XP beads (Beckman Coulter, Brea, CA, USA) were used for cleaning of the PCR products at all stages. For the second PCR Nextera XT, Index Kit (Illumina) was used adding unique sequencing adapters to the amplicons. Quantitative PCR was used for library quantification and pooled libraries were the sequenced using Illumina MiSeq. Upstream analysis of sequencing data was done using QIIME and UPARSE and downstream analysis using custom R scripts on samples with good quality reads (>5000). Concentrations of 27 cytokines were measured in vaginal swab samples using a Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad Laboratories Inc., USA). Assay plates were read using a Bio-Plex Suspension Array Reader (Bio-Rad Laboratories Inc., USA). Data were analyzed using Bio-Plex manager software (version 4). Cytokine levels that were below the lower limit

of detection of the assay were reported as the mid-point between zero and the lowest detectable level measured for that given cytokine.

Pregnant women in this cohort had vaginal microbiota that clustered into 3 main community state types (CST): 109/356 (31%) CST1 - *Lactobacillus iners* dominant, 102/356 (29%) CST2 - *Lactobacillus crispatus* dominant and 145/356 (41%) CST3 - *Gardnerella vaginalis* dominant. There was a high prevalence of dysbiotic vaginal communities and *L. iners* was the predominant taxa found in > 90% of the women. When exploring whether the vaginal microbiota is associated with pregnancy outcomes, *L. iners* was highly associated low birth weight (LBW) and small for gestational age deliveries (SGA) while in contrast *G. vaginalis* was associated with normal birth outcome. There were no strong relationships identified between preterm birth (PTB) and vaginal microbiota. There was a strong correlation between vaginal microbiota and proinflammatory and adaptive cytokines. BV associated organisms (*Gardnerella, Aerococcus, Prevotella* Coriobacteriaceae and *Dialister)* were highly inflammatory while *Lactobacillus* spp were associated with low inflammation. Low levels of IL-13 and PDGF-BB cytokines were associated with LBW and PTB. We found that HIV status was highly associated with high vaginal microbial diversity. HIV-infected women had significantly higher alpha diversity in their vaginal microbiota, and they were more likely to have CST3. Surprisingly, having a diverse community type was not associated with high levels of vaginal inflammation. However low levels of IL-13 and IL-I7 while high levels of TNF-α were associated with HIV status.

In conclusion this study found that a highly dysbiotic vaginal environment is characteristics of Zimbabwean pregnant women and that vaginal microbiota is weakly associated with some poor pregnancy outcomes. The high prevalence of *L. iners* and its association with poor pregnancy outcomes suggests that vaginal microbiota may partly explain the high incidence of adverse pregnancy outcomes in African women. Low level of Th2 cytokines and growth factors may lead to adverse birth outcomes. Longitudinal studies of vaginal microbiota and soluble factors are needed in African women.

Chapter 1: Introduction

1.1 Study Overview

Globally, the main direct causes of neonatal death are estimated to be preterm birth (PTB; 28%), severe infections (26%), and asphyxia (23%) [1]. Neonatal mortality accounts for 45% of under-five deaths in sub-Saharan Africa, and has not declined despite much effort the past decade [2]. Preterm delivery (PTD), intrauterine growth restriction (IUGR), and neonatal sepsis are responsible for approximately 99% of neonatal deaths that occur in low- and middle-income countries [3]. In Zimbabwe, preterm birth complications, birth asphyxia and neonatal sepsis account for 29% of under-five mortality [4]. Furthermore, HIV infection is the leading cause of childhood death in Zimbabwe contributing 22% of childhood mortality [4]. Among other factors that may influence infant mortality, the gut microbiota has been extensively studied and proven to be important in determining health. Likewise for the past decade the vaginal microbiota have been studied in different ethnic origins and geographical locations [5-9]. However, the bacterial diversity of the maternal vagina in health and disease has underexplored in African pregnant women.

The composition of vaginal microbiota in pregnancy is important as it may influence susceptibility to adverse pregnancy outcomes [10]. The shift of the vaginal microbiota caused by the replacement of protective resident microorganisms such as *Lactobacillus spp*. by anaerobic bacteria is termed bacterial vaginosis (BV) [11]. BV is an extremely common reproductive tract condition worldwide, with a reported prevalence of between 10 and 50% among pregnant women[12]. A high prevalence has been reported among African populations of between 20 and 50%, while it occurs in about 30% of the women in childbearing age globally[13-15]. BV is highly associated with several pregnancy complications such as preterm labour (PTL), neonatal sepsis [16], premature rupture of membranes (PROM), low birth weight (LBW) [17], preterm delivery (PTD) and possibly spontaneous abortion [18-20]. These complications have been found to be more prevalent in HIV-infected women compared to uninfected women, regardless of ART treatment [21, 22]. On the other hand ART regimens vary substantially in their association with some poor birth outcomes [23]. Since BV is prevalent in women of African descent, it has been demonstrated that more adverse birth outcomes related to BV occur in these women [5, 24-26].

A high prevalence of BV has been reported in Zimbabwe among HIV-infected (36%) and HIV-uninfected (26%) women of child bearing age [27]. Prevalence of BV in Zimbabwean pregnant women is reported at 32.6% [28]. To date there is no documented data on vaginal microbiota in relation to pregnancy outcomes in sub-Saharan Africa. Although vaginal microbiota has been studied in different populations, contrasting findings have been reported in different populations and ethnic groups, therefore the need to consider geographical diversity. Identification of specific organisms associated with adverse pregnancy outcomes could lead to interventions or diagnostic algorithms to prevent and predict these outcomes in this population.

It is with this background in mind that this study was conducted, with the overall goal of providing insight into the role that bacterial commensals and/or infections and resultant inflammatory responses play in adverse pregnancy outcomes in Zimbabwe. This was achieved through the use of next generation sequencing techniques which permit deconvoluting of microbial communities with greater resolution. The sequencing of the bacterial 16S ribosomal RNA gene broadens and deepens the scope of characterisation of bacterial communities. This study aimed to first improve our understanding of the burden of certain maternal and neonatal adverse outcome-related vaginal pathogens in Zimbabwe. We further aimed to examine the mechanisms by which these microbes may lead to adverse outcomes, including host immune and inflammatory responses. In a cohort of pregnant Zimbabwean women, we investigated potential organisms associated with adverse pregnancy outcomes and HIV.

1.2 Literature review

1.2.1 Pregnancy and neonatal morbidity and mortality

Pregnancy outcomes are key population and life expectancy indicators and are one of the major determinants of infant mortality. Infant mortality rate is an indicator of the health and wellbeing of a nation [29]. Poor pregnancy outcomes are experienced in developing countries more frequently than in developed countries, partly due to different social and economic factors that affect the health systems [30]. Worldwide, the early childhood mortality in 2017 was 4.1 million, of which 75% of all under-five deaths occurred within the first year of life [3]. Preterm neonatal mortality contributes significantly to the high incidence of death among neonates; The World Bank (2015) reported that 85% of the neonatal mortality occurring around the globe is contributed by preterm birth [31]. Furthermore, the World Health Organization (WHO) reported that the risk of a child dying before their first birthday was over six times higher in the African region (52 per 1000 live births), than in the European region (8 per 1000 live births) [3, 32]. This is attributable to neonatal causes (26%), child pneumonia (21%), malaria (18%), diarrhoea (16%), HIV/AIDS (6%), measles (5%), accidents (2%) and is exacerbated by the lack of health infrastructure [33], economic, geographic, and social factors [32, 34].

Medical advances in public health have helped increase the number of deliveries conducted in the health facilities worldwide, but despite this effort, the adverse birth outcomes in most geographical areas have not decreased [30, 35]. Infant mortality rate remains high and is a significant challenge in sub-Saharan Africa despite the UN "Millennium Development Goals" which sought to reduce this [36]. Infant mortality and adverse birth outcomes are also affected by racial and ethnic disparities between and within countries [37]. For example, in the United States, black and Hispanic women have the highest rates of preterm and small for gestational age (SGA) infant deliveries compared to their non-Hispanic white counterparts [37]. These findings generally agree across studies showing a higher risk of PTB and LBW in black populations compared to white [38-40]. Adverse birth outcomes such as PTB, still birth, SGA, PROM, and LBW cause more than 80% of neonatal deaths and intensify post-neonatal mortality risk and long term neurodevelopmental impairment [41, 42].

1.2.2 The vaginal microbiome

Neonatal and post neonatal mortality are often caused by infections, intrapartum conditions, and PTB complications, among other factors [41-43]. During pregnancy, gross metabolic changes occur which support growth and development of both the mother and the foetus [44]. Among these changes are shifts in the human microbiome, including the microbes housed in the vaginal canal. The vaginal microbiome exerts many beneficial homeostatic functions in the human vagina, although there are factors that can lead to changes causing vaginal dysbiosis [45]. Numerous studies have evidenced the influence of the vaginal microbiome on adverse birth outcomes, with disparate results [46-49]. Therefore, there is a need to explore these findings in different geographical areas and populations.

The vaginal microbiome consists of complex physiologic microbial communities composed of different niches with metabolic and genetic diversities [6]. Vaginal bacterial communities (microbiota) play an important role in maternal and neonatal health [48]. Vaginal dysbiosis, which is the imbalance of the vaginal microbiota where *Lactobacillus* spp. are replaced by diverse anaerobic bacteria, has been associated with poor birth outcomes [48] [46].

The human body hosts more than a billion microorganisms that reside on body surfaces and in cavities, forming ecological communities of microbial species [50, 51]. The human microbiome maintains a mutualistic relationship with the host, providing the first line of defense against infections [52]. Additionally, this relationship between the human body habitats and the diverse microorganisms is important for development, health and nutrition [53].

1.2.3 Healthy vaginal microbiota

A healthy vagina is described as consisting of a balanced symbiotic relationship between it and the bacterial community within. The host maintains this balance by reducing the number of bacteria through sloughing of the vaginal epithelial cells. However, the indigenous bacterial community feeds on the nutrients provided by glandular secretions and sloughed cells to replenish their numbers [54]. A healthy vagina is characterised by abundance of *Lactobacillus* species, which were first described by Albert Döderlein in 1892 as gram-positive, non-spore forming rods which

produced lactic acid that could inhibit the growth of pathogenic bacteria [55]. During this time, it was considered that any depletion or reduction of the *Lactobacillus* species was a sign of invasion by pathogenic bacteria that could cause diseases [55, 56]. However, recent studies have shown that the composition of the vaginal communities is not static, but instead, changes over time and undergoes shifts in representation, abundance, and virulence **(Figure 1.1)** [57, 58]. Furthermore, composition is also influenced by factors such as menstrual cycle, age, puberty, menopause, nutrition, ethnicity, geographical area, pregnancy and genetic factors, which are not yet fully understood[59], among others **(Figure 1.1)** [57]. The role of resident indigenous bacterial communities cannot be underestimated, as they play a crucial protective role in host resistance to colonization by exogenous pathogenic microorganisms [60].

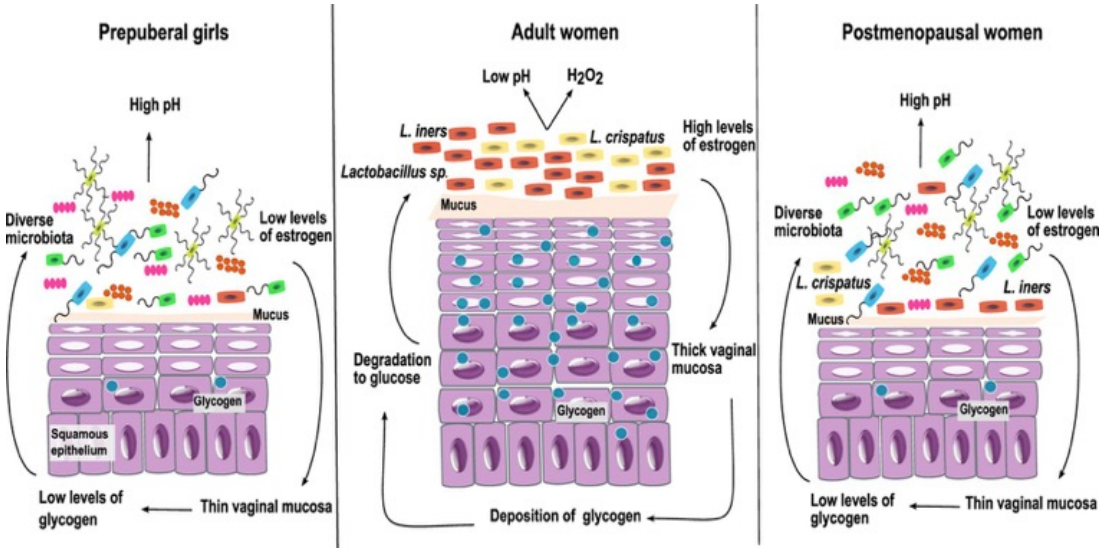


Figure 1. 1 Changes in the vaginal mucosae during different stages of a woman's life.

During prepuberty, the low levels of oestrogen result in thin mucosae and low levels of glycogen, which stimulate the growth of diverse microbial species. In adult stage of life, the glycogen levels increase, due to the increase in the levels of oestrogen. The degradation of glycogen to glucose selects for glucose-fermenting microorganisms such as *Lactobacillus* spp., which lower the vaginal pH and prevent growth of pathogenic bacteria. Post menopause, the levels of oestrogen once again decline, reducing the deposition of glycogen thereby selecting for a high diversity of bacterial species (Petrova . 2013).

1.2.3.1 *Protective role of Lactobacillus*

There are about 130 known species of lactic acid-producing *lactobacilli* and of these, about 20 have been detected in the vagina [59]. The most common species that characterise the vaginal microbiota of healthy non-pregnant and pregnant women are *L. crispatus, L. iners, L. gasseri,* and *L. jensenii* [10, 61]. *Lactobacilli* are known as protective resident microorganisms which possess various homeostatic properties that play a role in balancing the vaginal environment [11, 62]. They provide defence against infection, in part, by producing hydrogen peroxide and lactic acid, which maintains an acidic pH and inhibits growth and colonization by pathogenic bacteria [63, 64]. Furthermore, they also produce bacteriocins which inhibit the growth of omnipresent pathogens and biosurfactants which reduce adhesion of pathogens. Co-aggregation molecules also help to block the spread of pathogens [65, 66].

In the presence of high concentrations of oestrogen vaginal epithelial cells (VEC) produce glycogen which is fermented by *Lactobacillus* spp (*L. crispatus, L. gasseri,* and *L. jensenii)* to produce D- lactic acid isoforms. However, *L. iners* possess genes responsible for vaginal glycogen degradation and use it as a source of carbon [67], producing L-lactic acid isoforms [68]. This acid directly affects host immune responses and microbial interactions [69]. L-lactic acid has been evidenced to inhibit HIV while D-lactic acid inhibits some bacteria [70]. Overall, lactic acid production creates an acidic environment that inhibits the growth of a wide range of BV-associated bacteria while favouring survival of *Lactobacillus* spp [71, 72]. Additionally, vaginal acidity increases the trapping of the HIV-1 virions by the cervicovaginal mucus [73]. It has further been demonstrated that lactic acid can also affect host immune functions by inhibiting the production of pro-inflammatory cytokines including IL-6, IL-8 and IL-1ra associated with acquisition of HIV [74, 75] Alternatively, lactate can elicit an anti-inflammatory response from VEC [76]. In addition to the acidic pH created by the *lactobacilli* species, production of lactic acid, the production of H_2O_2, lysostaphin, and bacteriocins also drastically inhibit the growth of potentially pathogenic organisms such as *Escherichia. Coli (E. coli), Klebsiella, Enterococcus faecalis (E. faecalis), N. gonorrhoeae* [77], HSV-2 [78], *Staphylococcus aureu*s [79, 80] (Figure 1.2). However, recent studies have questioned the importance of H_2O_2 and found it less important especially under hypoxic conditions which are usually found in the vaginal environment [76, 81]. *Lactobacilli* produce less H_2O_2 in an oxygen deficient environment as compared to an oxygen abundant environment [81, 82]. Additionally,

H_2O_2 is inactivated by the high antioxidant capacity of cervicovaginal fluid and semen [72, 83]. Furthermore, recent findings have demonstrated that H_2O_2 is considered more bactericidal to vaginal *lactobacilli* viability than to17 tested BV associated bacteria species as compared to exogenous added H_2O_2 [72, 84].

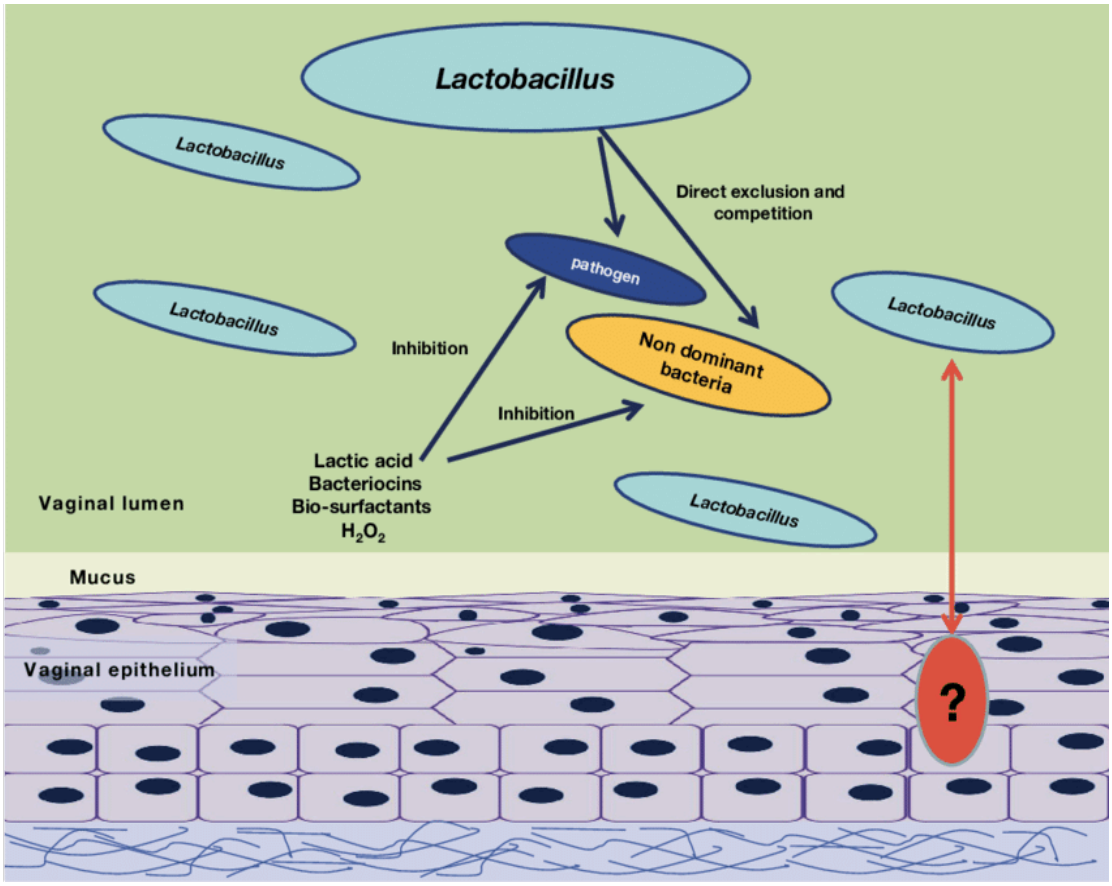


Figure 1. 2 Lactobacilli's protective role against vaginal pathogens

Lactobacilli produce mainly lactic acid and hydrogen peroxide (H_2O_2). The latter releases oxygen and has a disinfecting effect. Bacteriocins inhibit the growth of pathogens which are always present in the vagina. Biosurfactants cover the surface of the vaginal wall, inhibiting the adhesion of pathogens while co-aggregation molecules block the spread of pathogens (Sylvie Miquel *et al.,* 2015)

1.2.4 Bacterial vaginosis

Bacterial vaginosis (BV) is a vaginal dysbiosis characterized by high pH, and occasionally vaginal discharge [19]. A concomitant reduction in the quality or quantity of lactic acid producing *lactobacilli* results in an overgrowth of anaerobic bacteria, or microaerophilic species (BVAB1, BVAB2, *Megasphaera* spp., *L. amnionii*, *S. sanguinegens*, *Gardnerella vaginalis*, *Atopobium vaginae*) [85] (Esber, 2015 #51}. When symptomatic, BV clinically presents as malodorous and copious off-white, thin, homogenous vaginal discharge [13, 86]. However, a high proportion of BV cases are asymptomatic in both the general population and in pregnant women [19, 64] Additionally, asymptomatic BV is not treated according to current CDC guidelines, unless during pregnancy [87, 88]

Although BV is extremely common worldwide [12, 89, 90], prevalence is reportedly low in pregnant white women from Asian Pacific regions 11.1%, Colombia 9.6% and Canada 7.1% [91]. Conversely, a high prevalence of between 20 and 50% has been reported among pregnant African women [13-15], with a longitudinal study conducted on a sub-population of sub-Saharan African women (in Ethiopia, Kenya, Rwanda and South-Africa), finding a BV prevalence was 38% [25, 92]. BV prevalence is found to vary from country to country and may be dependent on risk factors. A study conducted in the United States including non-pregnant women of reproductive age showed lower rates of BV; 23% in white women compared to non-white women (51% in African-Americans and 32% in Mexican Americans) [89].

The epidemiology and pathogenesis of BV remain unclear, thus making it difficult to classify **(Figure 1.3)** [26]. It is thought that an imbalance in the vaginal environment is created when there is an increase or decrease in certain hormones such as oestrogen, or any other factor which alters microbiological balance [11]. Due to this imbalance, lactobacilli fail to metabolize glucose from vaginal glycogen into lactic acid. Anaerobic bacteria then transform glucose into fatty acids [93]. These fatty acids are favourable to the growth of potentially pathogenic bacteria and contribute to the development of BV [11, 93]. For example, during the postnatal period, the vaginal microbiome of 40% of women will be depleted of *Lactobacillus* spp. due to hormonal changes leading to dysbiosis [10]. Furthermore, the malodor that often accompanies BV is attributed to metabolism of amino acids by anaerobic bacteria, which produces biogenic amines [94] which degrade vaginal mucin [95, 96]. On another hand, a divergent conceptual model

by Schwerbek *et al., (*2014) and some epidemiological findings suggest that BV may be a sexually transmitted infection [97, 98]. Even though some studies have found BV to be associated with the prevalence and incidence of some sexually transmitted infections no clear evidence exists that it is a cause of STIs [99, 100]. Factors such as hygiene practices such as vaginal douching, social factors (multiple sexual partners) [101, 102] and fat rich diet, have been reported by various studies to be associated with BV development while vitamin A and folate are associated with decreased risk of BV [93, 103].

Pregnant women are generally more vulnerable to bacterial infections, since they are considered to be in an immunocompromised state. The fluctuating levels of steroid hormones (oestradiol and progesterone) [104] have been correlated to susceptibility to *Listeriosis* [105], gingivitis and pyogenic granulomas [97] which are strongly linked to the risk of preterm delivery [106]. As previously mentioned, BV has been associated with poor pregnancy outcomes such as PTB, still birth and PROM, and therefore BV diagnosis is imperative for the management of pregnancy in order to reduce the risk of poor outcomes.

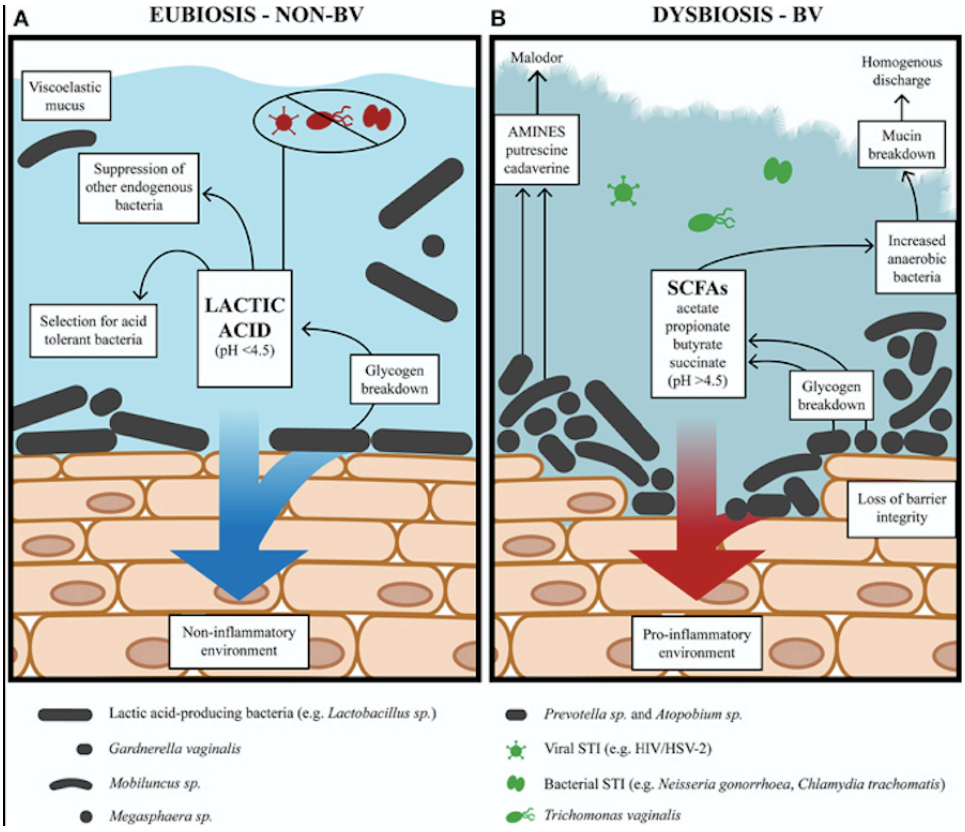


Figure 1. 3 The vaginal environment during alternative states of eubiosis and BV. **A.** During eubiosis, lactic acid-producing bacteria acidify the vaginal milieu pH <4.5 (average ~3.5) with

lactic acid as the predominant metabolite. Lactic acid potently inactivates STI while lactic acid-producers, such as Lactobacillus, generate a non-inflammatory environment. **B.** During BV, the vaginal environment has a lower redox potential conducive to the growth of diverse anaerobic bacteria and higher bacterial load. The concentrations of mixed SCFAs and amines also increase, and are accompanied by loss of vaginal acidity, pH >4.5. The diverse anaerobic bacteria generate virulence factors which compromise epithelial barrier integrity, degrade mucin, and generate a pro-inflammatory environment(Aldunate *et al.,*2015)

1.2.5 Diagnosis of BV

BV diagnosis is done using the Amsel criteria, Hay/Ison technique, Nugent scoring, or for places without laboratory facilities, the BVBlue [107] bedside point-of-care test.

Amsel criteria: This is a widely accepted as it is partly based on clinical symptoms. To be confirmed BV positive, 3 of 4 criteria should be met: 1) vaginal pH > 4.5; 2) clue cells in the vaginal fluid; 3) a milky, homogeneous vaginal discharge; 4) and the release of an amine (fishy) odour after addition of 10% potassium hydroxide to the vaginal fluid [108].

Hay/Ison technique: For this method, women are classified into 3 main groups according to the abundance of *Lactobacillus* and *Gardnerella* morphotypes only in a Gram-stained vaginal smear **(Table 1.1) [109]**.

Table 1. 1 The Hay/Ison classification for BV

	***Lactobacillus* Morphotypes**	***Gardnerella* Morphotypes**
Normal (group 1)	Many	Few
Intermediate (group 2)	Equal amount	Equal amount.
BV (group 3)	Few	Many

Nugent score: This is the gold standard for BV diagnosis and is based on Gram-staining of a vaginal smear and scoring by morphotypes per visual field: *Lactobacillus* morphotypes (large Gram-positive rods), *G. vaginalis* and *Bacteroides* species morphotypes (small Gram-variable and gram-negative rods) *Mobiluncus* species morphotypes (curved Gram-variable rods; **Table 1.2)** [110, 111]. Women are classified as normal (score: 0-3), intermediate (4-6), or BV (7-10).

Table 1. 2 The Nugent's scoring system: Morphotypes per visual field

Score	***Lactobacillus* morphotype**	***G. vaginalis* morphotype**	***Mobiluncus* morphotype**
0	>30	0	0
1	5—30	<1	1—5
2	1—4	1—4	>5
3	<1	5—30	
4	0	>30	

Scores: Normal = 0-3, Intermediate = 4-6, BV = 7-10.

BVBlue tests: Bacterial pathogens associated with BV such as *G. vaginalis, Mobiluncus, Bacteroides* and *Prevotella* produce an enzyme called sialidase. The BVBlue test is a chromogenic diagnostic test-based point of care tests which detects elevated vaginal fluid sialidase activity. Results are obtained in ten minutes where a

blue or green color indicate a positive result (increased sialidase activity) while a yellow color indicate a negative result (no increased sialidase activity).

1.2.6 Molecular analysis

Microscopy and culture dependent diagnosis can detect only limited types of organisms [56, 112]. These methods limit our ability to accurately assess the microbial world. Many microbial species are difficult to culture in the laboratory; therefore, characterisation of the human microbiota using these methods was not comprehensive [113]. However, recent advances in molecular methods such as polymerase chain reaction (PCR) and Next Generation Sequencing (NGS) have provided substantially more detail regarding the microbes present at mucosal surfaces [114, 115]. The advent of molecular techniques such as 16S rRNA gene deep sequencing for characterisation of the vaginal microbiome has led to a growing list of organisms that may constitute BV, differing from the culture based characterisation which named *Gardnerella* species as the main BV-associated organisms [57, 116, 117].

Molecular methods have shown that any combination of the following genera may be highly predictive of BV positive women: *Gardnerella, Mycoplasma, Atopobium, Mobiluncus, Prevotella, Sneathia, Leptotrichia, Peptostreptococcus*, and BV-associated bacterium 1-3 [BVAB1 to BVAB3 *(M. indolicus)*] [15, 63, 64, 85, 117]. Studies conducted in Europe show that *Gardnerella, Atopium vaginae, Prevotella, and Sneathia* are commonly prognostic of BV [118-122]. Furthermore, these studies strongly agree that the combination of *Gardnerella vaginalis (GV)* and *A. vaginae*, and a low abundance of lactobacillus spp. is highly predictive of BV [118-122]. However, the importance of culture methods cannot be ignored as it helps identify viable organisms. Culture methods are time and cost effective as the samples will need less manual labour compared to manual preparation of samples before DNA extraction and possibly batching samples before processing. The two methods however complement each other depending on the setting, clinical or research and workload. Additionally, shotgun 16S rRNA amplicon sequencing detects only bacterial species, while the microbiome is also comprised of other taxa, including fungi and viruses.

1.2.7 BV treatment

The CDC guidelines recommend the treatment of BV in pregnant and nonpregnant symptomatic women to alleviate signs of infection and reduce risk of STI infection. CDC recommends the use of mainly metronidazole or clindamycin cream with tinidazole as an alternative. There have been several trials that evaluated BV treatment during pregnancy to reduce adverse outcomes, however these studies have yielded contrasting findings. In some studies, treatment of BV in pregnant women at risk of preterm delivery reduced the risk of PTB [123, 124]. However, in other studies, treatment of asymptomatic BV did not reduce occurrence of preterm delivery [125, 126]. In a meta-analysis by Brocklehurst *et al*. (2013), antibiotic treatment effectively eradicated BV but the overall risk of PTB was not reduced. In a randomized controlled trial, Guise and colleagues (2001) reported no benefit to routine BV screening and treatment, finding that some subgroups at high risk would benefit whilst BV treatment increased the risk of PTB in other subgroups [127]. Currently there is no consensus on the benefits of screening and treatment of BV in pregnant women, as there is no sufficient evidence of reduction of adverse birth outcomes[128, 129]. However this may be because current BV treatment recommendations are inadequate and also that a full understanding of microbiologic, epidemiologic, and sociologic determinants of BV is imperative in order to develop effective treatment strategies for BV in pregnancy[87].

1.2.8 Vaginal Microbiota classification

Molecular analysis has revealed that different populations and ethnic groups possess varied vaginal microbial communities, leading to diverse classifications of vaginal communities [51, 130]. In a study of North American women using NGS, Ravel *et al., (*2011) classified vaginal bacterial communities into five main community state types (CST): CST I (*L. crispatus*-dominated*),* CST II (*L. gasseri*-dominated*)*, CST III (*L. iners*-dominated), CST IV (diverse), and CST V (*L. jensenii*-dominated) [51] **(Figure 1.4).** In this study, four CSTs were dominated by *Lactobacillus* spp*.,* while CST IV had a paucity of *Lactobacillus*. Similar to BV, CST IV was characterised by highly diverse microbiota with a mixture of facultative anaerobic bacteria such as *Prevotella, Dialister, Atopobium, Gardnerella, Megasphaera, Peptoniphilus, Sneathia , Eggerthella, Aerococcus, Finegoldia,* and *Mobiluncus.* According to this classification, CST I, II, and III, which are considered *"healthy",* were prevalent in Asian and white women, while CST IV, associated with diseases such as BV [131, 132], was found to be common in Hispanic and black women. It was initially concluded that Hispanic and black women were more likely to be unhealthy; however, several studies have subsequently shown that communities with a paucity of *Lactobacillus* are more common in women who are classified as having a normal microbiota [51, 56, 85, 133]. Since then, several African studies have found different CSTs depending on cohort characteristics [134, 135].

It has become clear that geography and /or genetics likely play a role in the microbial communities that dominate in the vagina. For example, *L. iners* is the dominant *Lactobacillus* spp. found in vaginas of women in Brazilian, Japanese, Belgian, and generally in black African women [5, 116, 133, 136], while *L. crispatus* dominated communities are common in Swedish, Germany, Turkish, and generally in white European women [136-138]. *L. gasseri* and *L. reuterii* are dominant in Indians and Bulgarian women **(Figure 1.5)** [5, 139-141], while both *L. iners* and *L. crispatus* are more prevalent in Chinese women [142]. However, it is important to note that there are some limitations in using CST for classification of vaginal microbiota, since CSTs are based on relative abundance in the study population whose results may not have external validity. Dominant species are used to name the CST, thus ignoring rare taxa which may be clinically important and may have impact on observed outcomes.

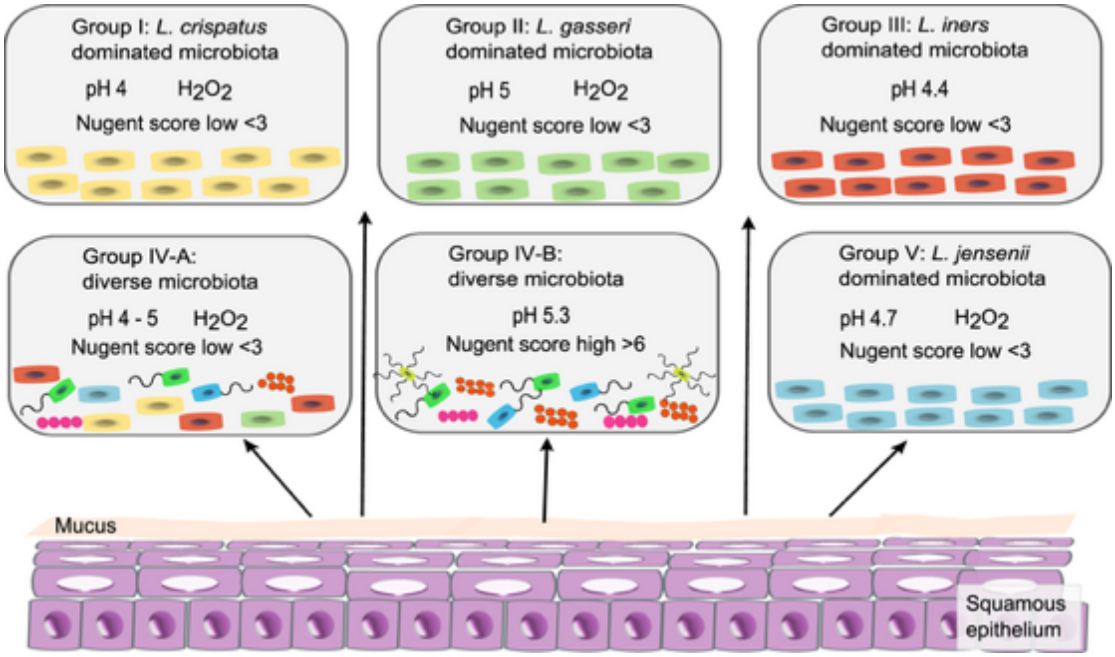


Figure 1. 4 Composition of vaginal microbiota in adult women as defined by Ravel *et al., (*2011)**.** Ravel *et al.,* (2014) sequenced the V1–V2 hypervariable region of the 16S rRNA gene of vaginal bacteria from North American adult women and described five community groups. Group I, II, III, and V are dominated by Lactobacillus spp., namely L. crispatus, L. gasseri, L. iners, and L. jensenii respectively, while group IV is diverse in composition. Community group IV can be separated into two subgroups – IV-A and IV-B. Community group IVA is characterized by the presence of L. crispatus or L. iners and strict anaerobes, while IV-B has a higher proportion of members of the genera Atopobium, Prevotella, Parvimonas, Sneathia, Gardnerella, and Mobiluncus. (Petrova *et al.,* 2013)

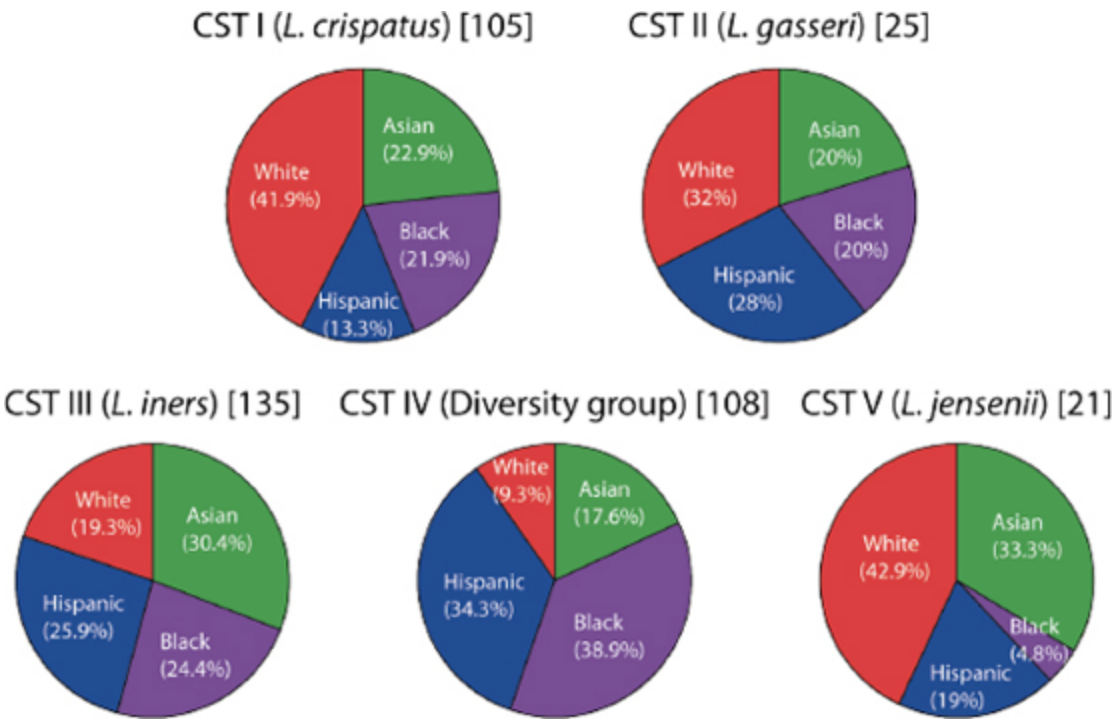


Figure 1. 5 Pie charts illustrating the proportions of each vaginal bacterial community group by ethnicity. The different distributions of dominant community state types (CSTs) in each ethnic group are displayed, with larger slices of the pies indicating the largest ethnic groups for each particular CST, while the smaller slices show the smallest ethinic groups for each CST. The numbers in square brackets show the sample sizes for each CST (Jacques Ravel and Larry J. Forney, 2011).

1.2.9 The vaginal microbiota of African women

Understanding population differences, common influential factors and the host and its interactions with the vaginal microbiota will help define normal or healthy vaginal environments [54, 143, 144]. The few studies that have been done in African women and women of African descent have shown that their vaginal microbiota is often dominated by *L. iners* and diverse communities commonly associated with BV [135, 145]. In a Tanzanian study, the core members dominating in every woman's vagina were *G. vaginalis* and *L. iners.* Contrary to the findings of Ravel *et al., (*2011), eight clusters of vaginal communities were identified in the Tanzanian cohort, in which two were dominated by *L. crispatus* and *L. iners*, where *L. crispatus* was associated with lowest pH and in the absence of *L. crispatus*, *L. iners* was used to predict low pH [145]. Furthermore, a study including young females from Durban, South Africa found four distinct community types which were termed cervicotypes (CTs) [135]. In women from Cape Town and Soweto, South Africa, Lennard *et al.,* (2017) found only three different vaginal community subtypes using fuzzy clustering of Bray Curtis distances [134]. Despite the use of different clustering techniques and varied definitions of a cluster, in all of these African studies, the diverse microbiota community types dominated, while a low prevalence of *Lactobacillus* community types was found [135]. Therefore, the "normal" vaginal microbiota for sub-Saharan Africa and women of African descent may be dominated by high diversity profiles, or if *Lactobacillus*-dominated, the most prevalent is *L. iners* [24, 57, 146]. *L. iners* is different from other common *lactobacilli* species and possesses genes that enable adaptation to different vaginal environments [24, 67, 147]. Longitudinal studies show that the vaginal microbiota of some women shifts between cluster types and depends on their factors such as sexual activity, menstrual cycle and oestrogen production [56, 130]. However, BV is present in every population, despite differences in microbial community types, and is detrimental to health particularly in pregnancy, with several studies demonstrating a relationship between poor birth outcomes and BV [18-20, 148].

1.2.10 The vaginal microbiome and birth outcomes

The vaginal microbiome harbours numerous bacteria that have important effects on health and disease states [149]. Reproductive health outcomes are likely to be influenced by the composition and interactions of the maternal vaginal microbiome throughout pregnancy [150]. A normal pregnancy is characterized by a highly stable

microbiome with low diversity dominated by *Lactobacillus* species; this stability is thought to be a result of the absence of cyclical hormones and the menstrual cycle, among other factors [61]. This is further demonstrated in a longitudinal study, done by Romero *et al., (*2014) which reported that vaginal microbiota was highly stable in North American women for the duration of pregnancy compared to their non-pregnant counterparts [58].

BV has been associated with several pregnancy complications such as preterm labour, PROM, LBW, preterm delivery [18, 20] and possibly spontaneous abortion [19, 148]. Additionally, BV may increase susceptibility to sexually transmitted infections such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis [64]*, *Trichomonas vaginalis [148]*, HSV-2 [151] and HIV [152-154] which are also associated with poor birth outcomes. A study by Gupta *et al.* (2013) showed that 23.5% of women with BV detected in early pregnancy <28 weeks had preterm births compared to 7% of those with normal vaginal microbiota [19]. A meta-analysis by Leitich *et al.* (2007) demonstrated that in pregnant women, asymptomatic BV doubled the risk of preterm delivery, as well as increased the risk of late miscarriages and maternal infections [155]. Additionally, in multiple studies, delivery of infants who were SGA or had LBW was significantly associated with symptomatic and asymptomatic BV [16, 17, 156]. BV carriage during early pregnancy increases the risk of fetal mortality between 14-24 weeks 6-fold and is a strong risk factor for late miscarriage [16, 155]. This concurs with reports in studies done in Danish pregnant women [49], Turkish pregnant women with a history of previous poor outcomes [47] and Nigerian pregnant women [18]. These findings suggest that the presence of BV in early pregnancy is more of a risk factor for adverse birth outcomes such as LBW, SGA, and miscarriage when compared to late pregnancy exposure.

It is postulated that specific organisms, rather than BV itself, may increase risk for adverse birth outcomes. For example, detection of *Mycoplasma hominis*, *G. vaginalis* and/or *A. vaginae* in isolation or in combination was associated with preterm delivery [157]. Foxman *et al.,* (2014) similarly reported that *Mycoplasma* was most strongly correlated with preterm birth followed by *Mobiluncus and A. vaginae* [158]. *U. urealyticum* and *M. hominis* are considered opportunistic pathogens and are suspected of inducing proinflammatory cytokines, which trigger pathways leading to preterm labour, PTD and pregnancy loss [159]. Moreover, Nelson *et al.,* (2014) found increased

abundance of BVAB1, *Megasphaera phylotype 1*, and *Leptotrichia/Sneathia spp.* in vaginas of women who reported having a previous preterm delivery [160]. However, Foxman *et al.,* (2014) reported that *Mageebacillus indolicus* (BVAB3) was associated with a reduction in preterm delivery risk among BV+ women and thus concluded that some BV-associated organisms are not associated with poor birth outcomes [158]. The concurrent presence of previously named *Bacteroides* (since assigned to *Prevotella* or *Porphyromonas*) and *M. hominis* in the vaginas of pregnant women was found to be associated with a risk of PTB and LBW [161], whilst in another study, the combination of *G. vaginalis* and *M. hominis* predicted the same outcomes [117]. However other studies have found conflicting evidence on preterm birth and association with vaginal microbiome. Stout *et al.,* (2017) found that, although no specific taxa were associated with preterm birth, a significant decrease in vaginal microbial community richness and diversity was associated with preterm birth [48]. Population and ethnic differences in the reported microbial causes of adverse birth outcomes and differences in methods used in analysis have made it difficult to establish specific etiologies.

1.2.11 HIV and the vaginal microbiome

HIV-infected women have a higher prevalence of vaginal microbial dysbiosis than the general population [56, 162]. Vallone *et al.,* (2012) reported that dysbiosis in pregnant HIV infected women worldwide ranged from 47% in Western countries, to 89% in African countries [14]. However, it is unclear whether this increased prevalence of BV is due to HIV, or is only a result of BV increasing a woman's chance of contracting HIV [24, 163]. In a study by Roberts *et al.,* (2012), BV prevalence was similar in the same women pre- and post-infection [164], suggesting the latter may be true.

Multiple studies and meta-analyses have demonstrated that BV is an independent risk factor for HIV acquisition [163]. While BV (defined as Nugent score ≥7) was found to be associated with a 1.6-fold increased risk of HIV acquisition compared to women without BV [165, 166], women with high diversity vaginal communities defined by 16S rRNA gene amplicon sequencing were predicted to have a 4-fold increased risk of acquiring HIV compared to their counterparts with *L. crispatus* dominant vaginal microbiomes [167]. Since BV is so prevalent, it is thought to be one of the major causes of incident HIV infections. For example, of the estimated 50% of HIV infections in a cohort of Ugandan and Zimbabwean women attributable to sexually transmitted infections and dysbiosis, 26% were attributable to HSV-2 infection, 17% to BV (Nugent score ≥7) and 12% to intermediate microbiota (Nugent score 4-6) [25, 168].

When examining specific taxa and the risk of HIV, in a recent study of five cohorts, McClelland *et al.,* (2018) reported that high relative abundance of the following taxa were strongly associated with HIV acquisition risk in women: *M. hominis, Parvimonas* spp*, Eggerthella* spp*., Gemella asaccharolytica, Sneathia* and *Megasphaera* and contrasted these same relationships with low relative abundance of *L. iners* [9]. In an African study, Gosman *et al.,* (2017) found that relative abundance of *Prevotella, Sneathia,* and other anaerobes were linked with increased inflammation and HIV infection [167]. A review done African studies by Passmore *et al.,* (2018) confirm the presence of higher vaginal diversity in women who later seroconverted, concluding that BV contributes to increased risk of HIV acquisition [152, 169].

Once a woman becomes HIV infected, there seems to be a relationship between BV and HIV RNA levels in genital tract samples [131, 170, 171]. Sha *et al.,* (2005) found a strong correlation between *G. vaginalis* and *M. hominis* counts in cervicovaginal

lavage and HIV-1 RNA levels in contrast to an inverse relationship with *lactobacilli* counts. This led to their conclusion that BV-associated vaginal microbiota influences genital tract HIV shedding [171]. *Propionibacterineae, Anaerococcus* and *Citrobacter* taxa were uniquely found in HIV-infected women in a study by Spear *et al.,* (2008) [172], while in a different study, it was found that BV+ HIV-infected women had increased microbial diversity compared to BV+ HIV-uninfected women, suggesting an association between HIV infection and genital microbial diversity [171]. This collection of findings would suggest that altered vaginal microbiota may be partly related to immune status **(Figure 1.6).** On the other hand, studies by Goel *et al.,* (2014), Bhattar *et al.,* (2011) and Micheletti *et al*., (2009) found no correlation between high HIV-1 plasma viral load and BV [173-175].

It seemed that *G. vaginalis* and possibly other microbes may reduce the efficacy of some forms of topical antiretroviral pre-exposure prophylaxis, since these organisms have been reported to metabolise tenofovir gel, leading to women with abundant *G. vaginalis* in their genital tracts using topical tenofovir being poorly protected from HIV infection [176]. This relationship may be restricted to tenofovir when used intravaginally, as a study by Heffron *et al*., (2017) found no significant difference in the efficacy of oral tenofovir pre-exposure prophylaxis between women with normal and abnormal vaginal microbiota [177].

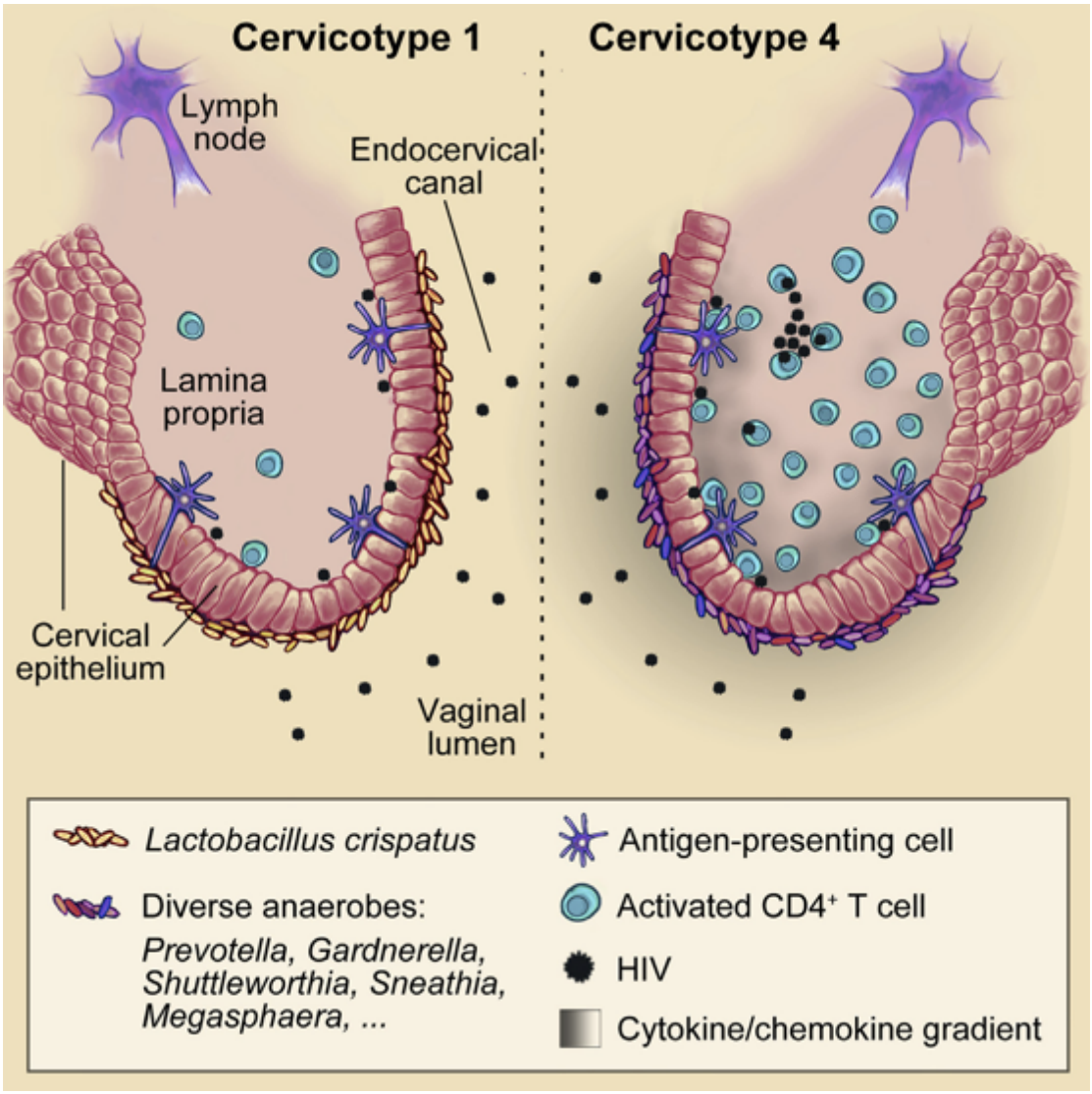


Figure 1.6 Vaginal microbiota and HIV susceptibility

Showing cervicotype 1 with more Lactobacillus crispatus and less CD4+T cells and cervicotype 4 showing a vaginal microbiota dominated with diverse anaerobes and increased number of CD4+ cells. Some vaginal microbial species are capable of increasing susceptibility to HIV infection (Gosmann *et al.,* 2017)

1.2.12 HIV, antiretroviral treatment (ART) and risk of adverse pregnancy outcomes.

Peripartum complications due to vaginal infections, which include chorioamnionitis, post-partum endometritis, and puerperal sepsis, are more frequent in HIV-infected women [14, 19, 27]. These complications may also increase the risk of mother-to-child transmission of HIV during pregnancy [12, 13, 152]. Gray *et al.,* (2007) observed that perinatal HIV transmission depends on various other factors, including the stage of disease in the mother, timely antenatal care and clinical management of labour and delivery [178]. Preterm birth, and delivery of LBW and SGA infants, among other adverse outcomes, are also associated with increased neonatal and infant mortality and morbidity in HIV-exposed uninfected infants [56, 178, 179].

However, studies have conflicting findings regarding associations between HIV and pregnancy outcomes, because some outcomes may be influenced by ART exposure. HAART which is considered as ART combination therapy and single dose therapies such as AZT and NVP, have also been studied in relation to pregnancy outcomes. In India, Patil *et al.,* (2011) did not find any association between LBW or SGA and HIV, in HIV infected women who were receiving extended-dose nevirapine, but on the other hand, there was a higher risk of poor birth outcomes in HIV-infected women who did not receive regular antenatal care compared to HIV-infected who received antenatal care [180]. In a systematic review, no convincing association between HIV status and birth outcomes was found in the different studies which were included [181]. In a South African cohort, Rollins *et al.,* (2007) found an increased risk of LBW and mortality in infants born to HIV infected compared to HIV uninfected women [182]. This study was conducted before the widespread availability of combination ART. Contrastingly, a recent study done comparing HIV infected pregnant women on ART and HIV uninfected pregnant women as controls, found no significant difference between HIV status and outcomes of PTB or IUGR although women who were HIV-infected were more likely to have PTB and IUGR as compared to HIV uninfected [183].

The use of certain antiretroviral drugs has been associated with adverse birth outcomes, which may confound the relationship between HIV status and birth outcomes. Goldstein *et al.,* (2000) noted a significant increase in the proportion of HIV-exposed neonates that were LBW, but an even higher proportion born to women who did not receive antepartum Studies conducted on ART/HAART [184] in Toronto, Ontario [185], Tanzania [186] and Côte d'Ivoire [187] found a high risk of delivery of LBW

infants associated with the use of ART in HIV-infected women. This is consistent with a meta-analysis conducted by Alemu *et al.,* (2015*)* which concluded that there was an association between HIV and use of HAART and adverse birth outcomes in developing countries [188]. In this meta-analysis, it was further found that the specific ART regimen may be influential, with protease inhibitors (PI) considered a greater risk factor than other regimens. The results of this study concur with the findings of studies conducted in Europe [189, 190], Germany and Australia [191], Latin America and the Caribbean [192], in which increased frequency of LBW infants and/or premature birth was found in women receiving PI-based therapy. In another study conducted in Western India, Darak *et al.,* (2013) found that women who received HAART even without PI had a higher risk of poor outcomes than those who received dual therapy with antepartum azidothymidine (AZT) with intrapartum nevirapine [193].

In studies including pregnant women in Europe, America and the Caribbean, no significant difference in pregnancy outcomes was found when comparing the effects of nucleoside reverse transcription inhibitors + non-nucleoside reverse transcription inhibitors (NRTI+NNRTI) versus NRTI+PI (protease inhibitors) regimens on birth outcomes [194]. Additional studies have found no association between HAART and birth outcomes [195, 196] There was no risk associated with use of tenofovir and efavirenz reported in studies including pregnant women from Uganda, Zimbabwe and Botswana [195, 196]. Similarly, in Nigerian [197] and South African studies, no association between HAART and poor pregnancy outcomes was found [198, 199]. Therefore, studies conducted in different regions show contradictory evidence of the potential risks of adverse pregnancy outcomes in HIV-infected women and in those using HAART. Putting all this analysis together we conclude that not only do the presence of certain vaginal bacteria or HIV status matter when considering the risk of adverse birth outcomes but also that the population being studied, and its geographical and social status may play an important role to be considered in these studies.

1.2.13 Vaginal innate immunity

Innate immunity refers to non-specific defense mechanisms naturally present in a host, and not due to prior sensitization to an antigen, whereas adaptive immunity refers to antigen-specific memory immune responses (in particular B and T cells). Although an oversimplification, in general, innate immunity involves all physical and chemical barriers such as skin, mucous membranes, blood, immune cells (phagocytic leukocytes,

neutrophils, dendritic cells, natural killer cells), and innate immune proteins and peptides (cytokines and antimicrobial proteins/peptides) which are activated by the presence of antigens [200]. Together, these components maintain a homeostatic environment that protects against disease and acts as the first line of defense against invading pathogens [201, 202].

The structure and function of the female genital tract (FGT) itself are important in innate immunity. The vagina is lined by non-keratinized stratified squamous epithelium, which consists of elongated and a nucleated basal cells that form a natural barrier to pathogens [203, 204]. The intermediate and superficial basal cell layers possess vacuoles containing considerable amounts of glycogen and mucin [205]. There are tight junctions composed of transmembrane regulatory proteins that prevent free movement of molecules across the epithelial sheet [206, 207]. Nonetheless, permeability is altered during an immune response to allow passage of effector molecules secreted by VEC [203, 207]. The upper layers of the stratified squamous epithelium are shed naturally every 4 hours; VEC also lose their tight junctions and detach from the basement membrane as a defense mechanism against pathogens that could have ascended and attached to the epithelial cells [208, 209]. Additionally, this shedding of VEC rich in glycogen is essential for provision of nutrients to *Lactobacillus* spp. Glycogen is metabolized by the VEC α-amylase to produce glycogen breakdown products such as maltose, maltotetraose and maltotriose, which *Lactobacillus* spp. are then able to utilise. Therefore, a decrease in this enzyme in the FGT may lead to a shortage of nutrients needed by *Lactobacillus* spp. and consequently to the depletion of *Lactobacillus* as seen in BV+ women [210, 211].

The VEC and mucins limit contact between the microbiota and host tissue, thereby contributing to antimicrobial activity [212]. VEC possess pathogen recognition receptors (PRR), such as toll-like receptors (TLRs), that recognize pathogen-associated molecular patterns. These receptors trigger cellular pathways leading to secretion of proinflammatory cytokines and antimicrobial peptides into the lumen [213]. This then attracts phagocytic and antigen-presenting cells which enter the epithelial cell layer by chemotaxis [214].

1.2.14 BV and inflammation

Although BV causes subclinical increases in proinflammatory mediators and is associated with adverse reproductive health outcomes that are mediated by inflammatory processes, BV is not widely considered a clinical inflammatory condition [215]. BV is a multidimensional process that involves numerous interactions between bacterial communities [38, 63, 216]. BV induces immune responses which result in elevated proinflammatory cytokine levels in the vagina and has been associated with several adverse birth outcomes such as PTD [217]. Specific BV-associated organisms have been implicated in alterations in immune responses that may result in unexpected pregnancy outcomes [64]. A number of studies have found elevated cervicovaginal cytokine concentrations when certain BV-associated species, such as *A. vaginae* [218], *Prevotella* spp.*, G. vaginalis* [219] and bacteria causing STIs such as *C. trachomatis* and *N. gonorrhoeae* [220, 221], were found to be colonizing the vagina.

Eades *et al*., (2012) demonstrated that IL-6, IL-8, G-CSF, IP-10, MIP-1β, RANTES, and GRO-α production by three epithelial lines were upregulated by BV associated bacteria as compared to commensal *lactobacilli* [222]. On the other hand, Kyongo *et al.,* (2015) observed that IP-10 concentrations were higher in cervicovaginal samples from women with *Lactobacillus* spp. dominance (except *L. gasseri)* compared to women with abundant *G. vaginalis* and *A. vaginae* [220, 223, 224]. These findings led the authors to suggest that BV-associated organisms may evade an immune response through tolerogenic mechanisms [223]. South African studies that included young women at risk of HIV demonstrated upregulated concentrations of multiple pro-inflammatory (Intereforn gamma (IFN-γ), tumor necrosis factor (TNF-α), Granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1α, IL-6, IL-8, IL-1β, IL-12p70) and anti-inflammatory (IL-10) cytokines in the presence of BV-associated microbiota [134, 135, 223] while other studies have found a down regulation of IP-10 [220, 221, 224]. A longitudinal study comparing cervicovaginal lavage of BV- and BV+ women where they measured subclinical vaginal inflammation (before and after treatment with metronidazole) reported that BV was associated with select soluble immune mediators and an increase in HIV target cells, thus potentially increasing susceptibility to HIV infection [225]. On the other hand, women with persistent BV had higher levels of intercellular adhesion molecule 1 (ICAM-1), IL-1β and TNF-α compared to women with resolved BV [225].

However, there is evidence that expression of certain cytokines in BV+ women may differ between individuals due to factors such as environmental influences and genetic polymorphisms [215, 226]. For example, BV+ women who possessed a TLR4 single nucleotide polymorphism(SNP) (rs1554973) had higher IL-1β than those without said allele [227]. Correspondingly, Genc *et al.,* (2002) in a case control study of pregnant women, investigated polymorphisms in fetuses of spontaneous preterm delivery and found that cytokine responses (e.g. TNFα and IL-1β) to BV-associated organisms were highly influenced by maternal genomic variations [228]. Pregnant women possessing a TNF-α polymorphism (TNFA-308G > A) in their blood had a higher expression of TNF-α in the presence of *G. vaginalis* and anaerobic gram negative bacteria compared to non-carriers [226, 229]. As a result, BV+ women with TNFA-308G > A polymorphism had a 6-fold increased risk of poor birth outcomes compared to BV+ women without this polymorphism [229]. It is thus important to investigate the specific organisms that are present in individual women and how these organisms interact with the vaginal host immune system since no single organism is present during all cases of BV and specific organisms may differentially influence the immune environment and pregnancy outcomes [230].

Proinflammatory cytokines such as interleukin (IL)-6, IL-8, Granulocyte colony-stimulating factor (G-CSF), Interferon gamma-induced protein (IP-10/CXCL10), Macrophage inflammatory protein (MIP-1β/CCL4), Regulated on Activation, Normal T Expressed and Secreted (RANTES/CCL5) and growth-related oncogene alpha (GRO-α) were found elevated in vaginal fluids during dysbiosis [208, 219]. However, in some studies, certain chemokines were found down regulated in genital secretions of women with BV [220]. Furthermore, in a review by Liebenberg *et al.,* (2017) microbial diversity has been found to be associated with increased inflammatory cytokines and cervical HIV target cells [231] however, in contrast, Lennard *et al.,* (2018) did not find an association between microbial diversity and HIV target cells [134]. These changes in immune mediators and immune cell populations may contribute to the increased risk of HIV associated with BV, as well as increased risk of poor pregnancy outcomes.

1.2.15 Specific vaginal microbes and innate immunity

1.2.15.1 *Lactobacillus spp.*

Homeostasis in the gut is maintained and controlled in part by the presence of lactic acid-producing bacteria which mitigate inflammation and preserve the barrier function (e.g. gut *lactobacilli* prevent microbial translocation during HIV infection) [40, 232]. Likewise, a number of studies have reported that the vaginal microbiota play an essential role in immune regulation in the vaginal mucosa [214, 233, 234]. Given the general agreement that *Lactobacillus* spp. are protective in the FGT, it follows that vaginal microbiota dominated by *Lactobacillus* spp. are associated with health, low proinflammatory cytokine production and normal birth outcomes **(Figure 1.7)** [235, 236].

Women who have *L. crispatus* and *L. jensenii* in their vaginal microbiota generally exhibit a negative association with cellular inflammatory markers [237]. A high percentage (83.3%) of women who had no *Lactobacillus* spp. present in their vaginal fluid were found to have a high vaginal IL-8 concentrations and these women were associated with a high risk of premature delivery [236]. However, it is becoming recognised that not all *lactobacilli* are the same. Generally, *L. crispatus and L. jensenii,* which are considered to be the optimal *Lactobacillus* species, are associated with good birth outcomes. They do not stimulate significant proinflammatory cytokine production in vitro and tend to dampen expression of IL-6, IL-8 and TNF-α in VEC in the presence of exogenous TLR antagonists [203, 218].

L. iners concentration was found to be positively correlated to SLPI, a known anti-inflammatory molecule that is present at minimal levels in a vaginal dysbiotic state, while *L. gasseri* was associated with high IL-1β in vivo [238, 239]. By mechanisms not yet established, *L. iners* and *L. jensenii* stimulate PRR, but this does not lead to an increase in proinflammatory cytokines IL-8 and IL-6 [240]. Jespers *et al.,*(2017) observed that *L. iners* abundance was significantly positively associated with IP-10 and IL-8, while negatively associated with IL-1α concentrations, in the FGT [116]. *L. iners* has been reported to express CRISPRs system related genes, which may play a role in its adaptability and response to altered environmental load and furthermore, it has an ability to express cholesterol dependent cytolysin and metabolic enzymes related to glycerol & mucin transport under BV positive condition [241]. Consequently this has

led to the conclusion by some that *L. iners* could be marker of vaginal dysbiosis and could be associated with BV-causing bacteria [242]. Although relative abundance of *L. iners* are lower in BV- women, *L. iners* is however found in both BV+ and BV- women showing their adaptability. *L. iners* has a small genome indicative of a symbiotic lifestyle unlike other *lactobacilli* and that the genes they possess encode for factors that are niche specific [243]. However, it has been reported that *L. iners* may not prevent HIV penetration through the cervicovaginal mucosal barrier, as opposed to *L. crispatus* which reinforces it [244]. Production of IL-1β and IL-8 by VEC is reported to be enhanced by lactic acid, showing the complexity of the synergistic relationship between microbiota and immunity [74]. While in contrast, a recent study found evidence that lactic acid has anti-inflammatory effect on the female lower genital tract inhibits pro-inflammatory mediators associated with susceptibility to HIV [76].

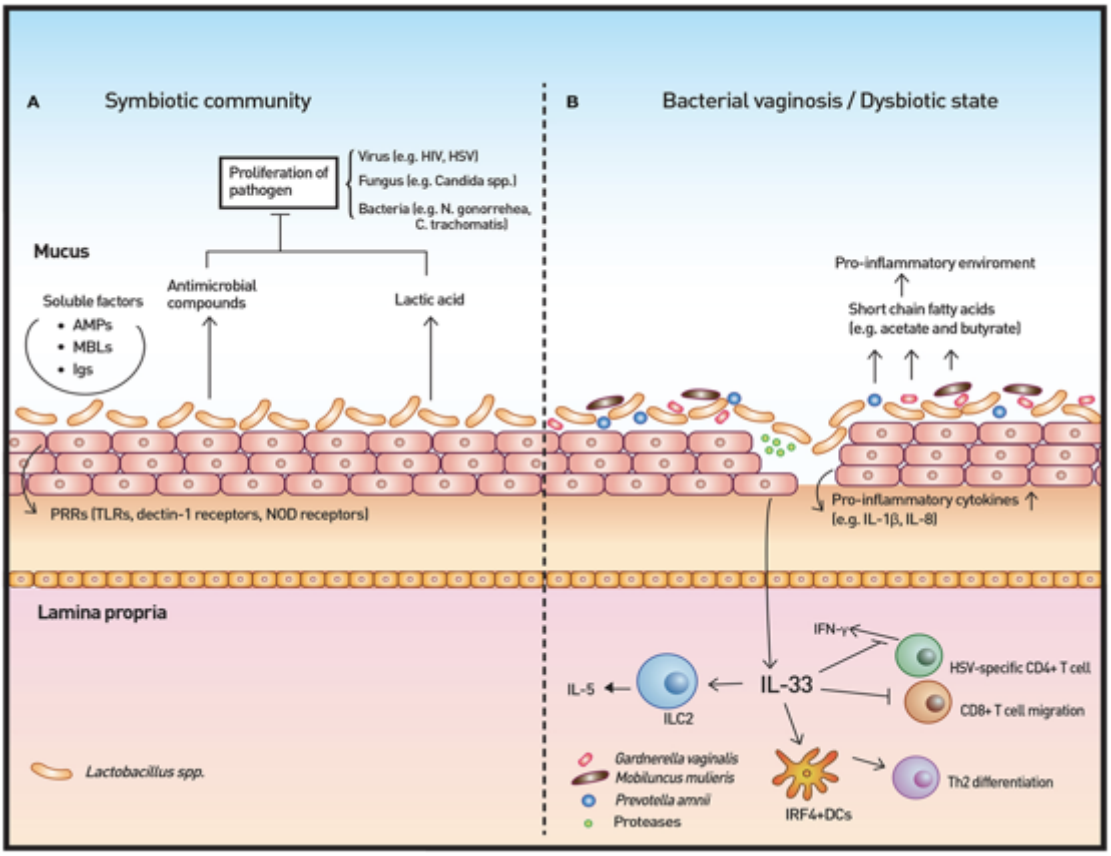


Figure 1. 7 Vaginal microbiota and immunity: **A** The vaginal microbiota in a healthy individual is dominated by *Lactobacillus* spp. The *Lactobacillus* spp. produce lactic acid, as well as antimicrobial compounds to control the growth of microbes. Other soluble factors, such as antimicrobial peptides (AMPs), mannose-binding lectins (MBLs), and immunoglobulins (Igs), contribute to the homeostatic immunity of the vaginal surface. In addition, the surveillance of commensals and pathogenic microbes is achieved by pattern recognition receptors (PRRs). **B** In cases of disrupted vaginal microbiota, such as bacterial vaginosis, community state type IV type microorganisms dominate to initiate an inflammatory response. Short-chain fatty acids produced by these microorganisms are likely to induce the production of proinflammatory cytokines. IL-33 has recently been identified as the key cytokine in association with antiviral immunity modulation by the vaginal microbiome. IL-33 is also responsible for the Th2-type immune response elicited by proteases that are secreted by pathogenic microbes (Park, Y. J. and Lee, H. K., 2017)

1.2.15.2 *Gardnerella vaginalis (GV)*

GV was named by Garner and Duke in 1953 when it was described as an etiological agent of non-specific bacterial vaginitis [245]. It belongs to the family Bifidobacteriaceae. It is a small, non-motile, non-capsulated gram variable organism that grows best in carbon dioxide. It is catalase, oxidase and beta glucosidase negative, whilst it ferments a wide range of sugars except for sorbitol, mannitol, rhamnose and melibiose.

The surface of the cell wall of GV is covered by fimbriae that are responsible for attachment to VEC both *in vitro* and *in vivo* [246, 247]. Patterson *et al.,* (2010) describes GV as the most virulent BV-associated organism since it contains three virulence factors, has biofilm formation and adhesion capabilities, and produces cytolysin [247]. *Gardnerella* is associated with pathogenicity because of possessing vaginolysin activity and an ability to form a scaffold for bacterial biofilms [246, 248]. A biofilm is formed when bacterial cells adhere to the host surface and to each other and produce a slimy extracellular matrix composed of a polymeric substance. This protects the bacteria and assists with immune evasion and resistance against antibacterial activities, causing chronic disease or recurrent infections [249]. When analysing GV biofilms, a number of other organisms were found to be incorporated within the biofilm layer [250].

GV causes inflammatory responses, with studies showing upregulation of IL-8, SLPI and RANTES *in vivo* and *in vitro* in response to GV [251.]. Furthermore, the cytoskeleton protein, vimentin, which supports cell strength and tissue integrity, was upregulated in VEC within eight hours of exposure to GV, while tight junction proteins (ZO-1 and ZO-2) were downregulated [252] **(Figure 1.8)**. GV has over 95% positive predictive value for BV when molecular based techniques are compared to Gram stain or Nugent score [120] and over 95% of women with recurrent BV had biofilms composed of GV and *A. vaginae* [249, 250]. Danielsson *et al.,* (2011) attributed the resistance of *Gardnerella* to H_2O_2, lactic acid and metronidazole to its ability to form biofilms [252, 253].

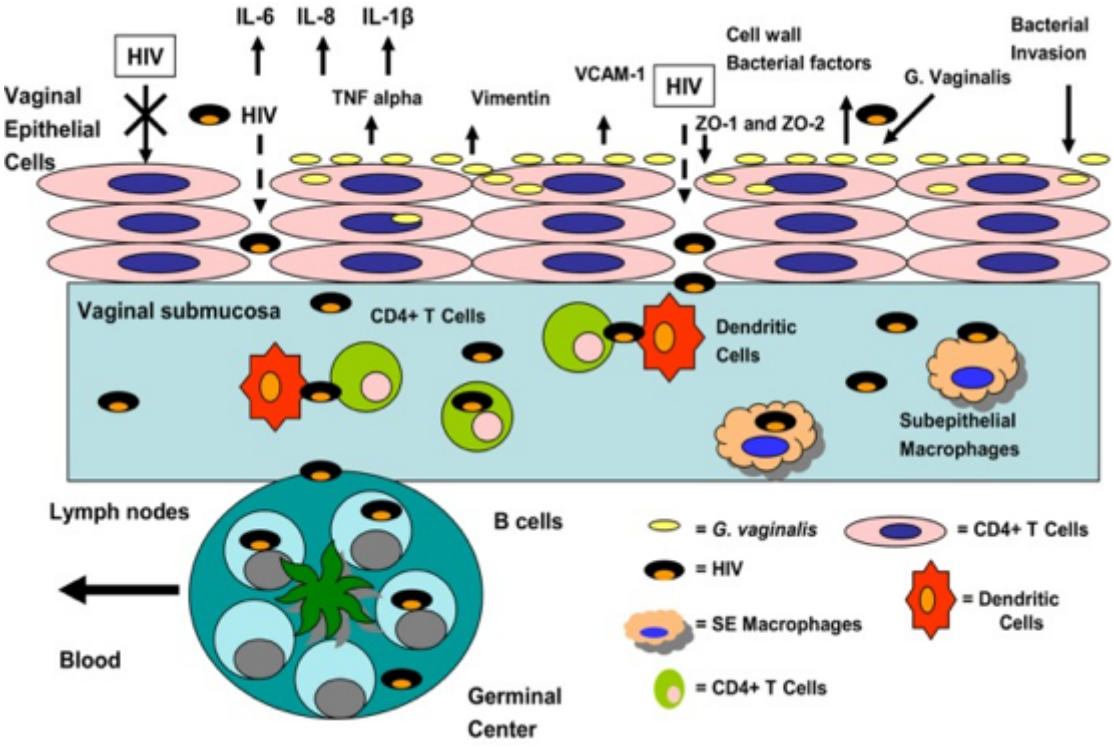


Figure 1. 8 Response in to *Gardnerella vaginalis* in vaginal epithelial cells

Exposure of vaginal epithelial cells to *Gardnerella vaginalis* results in upregulation of proinflammatory cytokines, namely IL-6, IL-8, TNF-α, and IL-1β. Vimentin is upregulated after *G. vaginalis* exposure. Upregulation of vimentin influences uptake and internalization of bacteria. Tight junction (TJ) proteins ZO-1 and ZO-2 are downregulated in vaginal epithelial cells exposed to *G. vaginalis*. Downregulation of TJ proteins could result in HIV passing in between cells (paracellular transport) *en route* to the vaginal submucosa where subepithelial T cells and macrophages reside and are highly permissive to HIV infection. Trafficking of infected T cells by resident lymph nodes would facilitate HIV dissemination via the blood (adapted from Alcendor 2016).

1.2.15.3 *Atopobium vaginae*

A. vaginae is a fastidious anaerobe gram positive coccus, which occurs as a single cell or in chains. It has a variable morphology, though in culture its usually masked by other coliforms. It is capable of producing large amounts of lactic acid [254], which makes its role uncertain.

A. vaginae has been identified in more than 90% of BV+ women compared to 12% in BV- women [118], and in 50% of women with gynaecological disorders [139]. However, although it has been isolated from BV- women, its high prevalence in BV may qualify it as a marker or predictor of BV [120]. *A. vaginae* correlates well with three out of four of Amsel's criteria and has been found together with GV in 75% to 98% of BV samples compared to less than 10% in BV- women [255]. Through qPCR, 16S rRNA amplicon sequencing and microarray techniques, GV and *A. vaginae* have been found associated with biofilm formation in more than 80% of BV cases [85, 256, 257].

A. vaginae accounted for 1-40% of the total mass of biofilms adherent to the vaginal mucosa in 70% of samples from BV+ women [249]. Therefore, biofilm formation could be the major reason why *A. vaginae* is resistant to metronidazole and is found in most BV recurrent cases [117]. In vitro, *A. vaginae* notably enhances proinflammatory cytokine, chemokine and antimicrobial protein expression by VEC, including *IL-8* [222], MIP-3α (CCL20) [218], RANTES (CCL5) [251], HBD-2, and IL-1β, as well as TLR2, MyD88 [258] and NF-κB [259] signaling pathways. Moreover, *A. vaginae* elicits consistent signatures associated with disruption of the vaginal epithelial membranes during innate immune responses [218]. *In vitro* models have observed high levels of TNF-α, HBD-2, IL-6, IL-8 and HBD-4 following *A. vaginae* stimulation [222, 258]. These studies agree with clinical studies that confirmed the same upregulations associated with GV and *A. vaginae*, with the latter notably the most abundant taxa in South African women with the highest levels of cervico-vaginal inflammatory mediators [135, 223].

1.2.15.4 *"Bacteroides" group (Prevotella and Porphyromonas)*

What were once thought to be *Bacteroides* morphotypes on gram stain for Nugent score classification were found to be *Prevotella* and *Porphyromonas* in a deep sequencing study conducted by Srinivasan *et al*., (2012)[85]. These organisms are gram negative

pleomorphic, anaerobic and non-motile rods. In many studies, *Prevotella* spp. have been found in most women regardless of Nugent score but relative abundance significantly increases with increasing Nugent score [156, 255, 260]. *Prevotella* produce polyamines during normal metabolic activity which produces a characteristic fishy odour associated with BV. The produced amines increase vaginal pH and create an environment that stimulates GV and *Peptostreptococcus anaerobius* growth [261, 262].

Prevotella spp., particularly *P. bivia* and *P. disiens,* produce fibrinolysin which disrupts mucosal surfaces, while sialidase and prolidases cause exfoliation of VEC [242]. *Prevotella* spp., particularly *P. bivia,* have also demonstrated the ability to prominently modify the cervicovaginal mucosal environment in an *in vitro* model by stimulating the production of MIP-3α, RANTES, IL-8, and IL-1β, and activating the proinflammatory transcription factor NF-κB [218, 251]. These studies concurred with *in vivo* studies conducted by Hedge *et al.,*and Jespers *et al.,* (2017) in which *P. bivia* was associated with higher levels of proinflammatory cytokines IL-1α, IL-1β, IL-8, MIP-3α and TNF-α and negatively associated with IP-10 in African women with both intermediate microbiota and with BV [116, 263].

1.2.15.5 *Anaerobic cocci: Peptostreptococcus.*

Peptostreptococci are anaerobic cocci that have been grouped with newly proposed genera like *Anaerococcus*, *Parvimonas*, *Finegoldia*, *Peptoniphilus*, and *Gallicola* [264]. Zozaya *et al.,* (2010) found *Peptostreptococcus* spp. in almost all specimens from BV+ women [255]. Onderdonk *et al.,* (2016) found increasing *Peptostreptococcus* abundance with increasing Nugent score [117], and found that these bacteria have an ability to form biofilms, however are unable to adhere to epithelial cells [247]. On the other hand, *Peptoniphilus,* which is a butyrate-producing genus with the ability to adhere to vaginal epithelial cells, was found to persist in women who failed BV treatment [265]. This genera is generally resistant to metronidazole, penicillins and clindamycin [255].

1.2.15.*6 Megasphaera*

Several studies have found that *Megasphaera* is highly associated with BV [90, 266, 267] in fact, is one of the four main BV genera [248]. In an *in vivo* study measuring

cytokines and microbial peptides, Megasphaera spp were found inversely associated with IL-8 quantities [219], while in another study, *Megasphaera* was found associated with high salisidase activity in BV [268].

1.2.15.7 *Sneathia*

Sneathia is the new name that was given to *Leptotrichia* in 2012 [269]. *Sneathia amnii* was found in more than 7% of women with BV [246]. In a separate study, *Sneathia amnii* was found prevalent in both BV+ and BV- women with a strong association with sexual activity [246, 248]. However, *S. amnii* was highly associated with mostly clinical signs and symptoms of BV; therefore, is implicated in BV pathogenesis [85].

S. amnii is able to produce collagenase and fibrinolysin which degrade VEC and promote their detachment [242]. *S. amnii* possess a number of virulent, cytotoxic factors such as potential invasins and adhesins, proteins involved in degradation of sialylated proteins and haemolysin [246]. In an *in vitro* study, *Sneathia* spp. were found to upregulate IL-1α, IL-β, TNF-α, and IL-8 [135].

1.2.15.8 *Mobiluncus*

Mobiluncus are motile fastidious, gram variable rod-shaped bacteria and are made up of two main species: *Mobiluncus mulieris* and *Mobiluncus curtisii. M. mulieris* is genera is said to be common in healthy women, while *M. curtisii* has been highly associated with BV [270-272]. The association of the *Mobiluncus* with BV has been established in numerous studies identified both by Nugent score and molecular techniques [273-275], and may be responsible for some of the malodor of BV [242].

Mobiluncus has the ability to produce both sialidase and mucinase in the FGTs of women with BV, which suggests the use of mucin as a substrate and may result in the depletion of this protective layer [276]. Culhane *et al.,*and Hedge *et al.,* (2006) found an upregulation of cervicovaginal sialidase, but not IL-1β and IL-8 in women with abundant *Mobiluncus* [263]. However, in an *in vitro* study, stimulation of immortalised human VEC with *Mobiluncus* culture isolates was associated with a significant upregulation of proinflammatory cytokines including IL-1α, IL-1β, TNF-α, and IL-8 [135]

1.2.15.9 *Mycoplasma and Ureaplasma*

Mycoplasma and *Ureaplasma* are facultative anaerobic organisms without a cell wall which replicate in a parasitic manner [117]. Therefore, they have been missed with Gram staining due to lack of cell wall and its associated structures which are difficult to stain. *Mycoplasma hominis* is the main *Mycoplasma* species associated with BV and poor birth outcomes [277]. It was isolated in 93(43%) of non-pregnant women with BV, while it another study it was found highly associated with risk of PTB regardless of cervical length [158, 277]. *M. hominis* and *Ureaplasma urealyticum* were detected in the vaginas of 75% of BV+HIV-infected pregnant women in Gauteng, South Africa [278]. *M. hominis* has been found in several studies in a symbiotic relationship with STIs [279]; it localises itself inside the *T. vaginalis* protozoa [280] where it replicates, thereby protected from host immune responses and antimicrobial treatment [281]. Although *U. urealyticum* colonisation was found to be higher in BV+ women in some studies [156, 282], others have found no significant differences in U. urealyticum colonization between BV+ and BV- groups [283].

U. urealyticum has the ability to produce enzymes, such as elastase and IgA protease, that compromise mucosal immunity. In addition it contains hydrolytic enzymes, urease and phospholipase C, that convert urea to cytotoxic ammonia which enhances the symptoms and pathogenesis of BV [242]. It also has the ability to induce the production of proinflammatory cytokines TNF-α and IL-6 by macrophages [284]. *M. hominis* possesses unique adhesins called TLR2-dependent, macrophage-activating, P50-related adhesins which may play an important role in triggering the inflammatory cascade [285].

1.2.15.10 *Dialister*

In a study conducted in North America, *Dialister* micraerophilus was found in almost 80% of women with BV [85] and is more likely to colonise African American and women of African descent [5] than Caucasian women. Virulence factors and the role played in BV have not yet been explored.

1.2.15.11 *BVAB1 to BVAB3 (Mageeibacillus indolicus)*

BVAB1, 2 and 3 have been recently described and are classified under the phylum *Clostridium* [267]. Molecular identification has shown 80% nucleotide identity with

Mobiluncus [117]. The three bacteria have been detected in >90% of women with BV and with high Nugent scores [267, 286]. BVAB3 (gram positive rods), renamed to *Mageeibacillus indolicus* [287], has been found to be associated with cervicitis [288]. Lennard *et al.,* (2018) found that BVAB-1 was highly inflammatory in young women at high risk for HIV infection [134]. However, Zozaya-Hinchliffe *et al.,* (2010) did not find any association between BV and BVAB1 despite higher abundance found in specimens from BV+ women [255]. The role of the BVAB group of organisms is thus unclear and further studies to explore these organisms are still needed.

1.2.15.12 *Streptococcus agalactiae, a vaginal pathobiont during pregnancy*

Streptococcus agalactiae or group B streptococcus (GBS) is a gram-positive encapsulated coccus, that is usually present as a single coccus, but can be present in chains. GBS is catalase negative and Christie-Atkins and Munch-Peterson-(CAMP) test positive and colonises the gastrointestinal tract, as well as the female genitourinary tract, intermittently [289, 290]. Colonisation can be intermittent, transient or persistent in individual women but the factors that determine colonisation are unclear [291]. Recto-vaginal GBS carriage prevalence worldwide ranges from 10% to 40% in healthy pregnant women [292, 293]. According to the WHO, Africa has the highest burden of GBS, with 54% of all estimated global cases, while 65% of stillbirths and infant deaths in Africa are due to GBS [3, 294, 295]. Studies in different settings have reported vertical transmission between mother and offspring of 29-85%, depending on the burden of colonisation in untreated mothers [296, 297]. GBS has been associated with adverse birth outcomes, including LBW [291], early and late onset sepsis and meningitis in neonates [297, 298], still birth [299], PTB [45] and PPROM [300]. However, in an study of over 1000 African women performed in urban and rural settings in pregnant women, GBS colonisation was not associated with poor birth outcomes [301]

Few culture independent studies have examined the relationship between GBS and vaginal microbiota in Africa [302, 303]. However, no significant relationship between the overall vaginal microbiota and GBS colonisation was found in non-pregnant women; although, positive trends toward significance were observed for *Staphylococcus*, *Corynebacterium* and *Aerococcus* taxa [304].

GBS has the ability to adhere to mucosal surfaces using factors such as surface serine proteins [290, 305], pilus proteins, alpha C proteins [306]and bacterial surface adhesins (BsaB, BsapA, BibA), which are also linked to GBS pathogenesis [307]. Some serotypes of GBS express β haemolysin/cytolysin and carotenoid pigment which influence cervicovaginal adherence and vaginal persistence [308]. GBS has 9 polysaccharide serotypes which have been described [309] and among these serotypes GBS serotype (ST) 17 strongly produces biofilm in an acidic environment *in vitro,* whereas in neutral environment, ST17 and ST19 only weakly produce a biofilm [310].

However, studies done using *in vivo* models have not been able to confirm the formation of biofilms by GBS [311].

GBS possesses α2,3-linked terminal sialic acid which mimics a critical host glycan and confers the ability to evade innate immunity [312] as it is identical to a host cell epitope expressed on glycoproteins and glycolipids on mammalian cells. This GBS molecule is identified as self-epitope and causes sialic acid-binding immunoglobulin-like lectins (Siglecs) to downregulate macrophage and neutrophil release and action [313, 314].

1.2.16 Inflammation and pregnancy outcomes

The maternal immune system during gestation is highly tolerogenic in order to prevent loss of the growing semi-allogeneic foetus in the womb [315] [316, 317]. Inflammation during pregnancy is thought to alter the homeostatic environment required for foetal growth leading to poor birth outcomes [318]. The presence of a stable microbiota and low vaginal pH during pregnancy possibly contributes to a state of relative immune quiescence [69, 209]. Preterm labour is thought to be a result of inflammation or imbalance in this tolerogenic state [319]. Most studies investigating the relationship between cytokines and pregnancy outcomes have been done in cord blood, amniotic fluid and maternal plasma/serum, while few have investigated cervicovaginal fluid. During maternal infection, the production of plasma cytokines such as interferon gamma and tumor necrosis factor alpha results in elevated prostaglandin levels that causes early labour [320]. Women who experienced complicated pregnancies, including cases of preeclampsia, PROM, PTD and spontaneous miscarriage, had high levels of serum cytokines throughout pregnancy, such as TNF-α [321], IL-6 and G-CSF [322], compared to women who had normal pregnancies.

In two studies performed by Romero *et al*., (1992) including pregnant women recruited between 1989 and 1992, IL-6 was found to be elevated in the amniotic fluid of those in preterm labour and those who delivered preterm neonates [323-325]. IL-1 was hypothesized to have played a role in initiation of preterm labour associated with intra-amniotic infection [324], and TNF was significantly higher in patients with PROM and premature labour [325]. This concurs with other studies that report that IL-8 and TNF-α polymorphisms in placentas, and IL-1α and/or IL-1β concentrations in vaginal fluid, are associated with spontaneous preterm delivery [326, 327]. In African and Hispanic

women, Genc *et al.,* (2002) found associations between spontaneous preterm delivery and foetal carriage of a mutation in IL-1 gene complex [228].

Increased vaginal concentrations of TNF-α or IL-1β in BV are hypothesized to stimulate amniotic membrane degradation or myometrial contractions to initiate the process of early labour, increasing the risk of preterm delivery [327]. This agrees with studies conducted in nonhuman primate models in which IL-1β and TNF-α were inducers of preterm labour, but not IL-6 or IL-8 [328]. IL-6 and IL-8 have however been known to cause some inflammatory changes in the foetal membranes [329]. Since BV is an inflammatory condition, it is therefore not surprising that it may be a cause of preterm labour.

Ethnicity may have an influence on the relation between cytokines and pregnancy outcomes. Velez *et al.,* (2008) observed differences in the relationship between amniotic fluid IL-1β, TNF-α, sTNFR1 and sTNFR2 concentrations and PTB in African American versus Caucasian women [330]. In African American women, IL-1β was associated with PTB, while this relationship was absent in Caucasian women. Therefore, there might be different mechanisms leading to PTB in different ethnicities

In a study conducted by Wilkinson *et al.,* (2017) increased TNF-α in maternal plasma of HIV infected mothers was highly associated with early delivery and LBW, while elevated umbilical cord IFN-γ and IL-12p70 concentrations were associated with significantly lower birth weights [331]. In several studies in HIV negative women, positive associations between serum proinflammatory cytokines (IL-1β, IL- 6, and IL-8) and PTB were found [332-334]. Similarly, IL-8 concentrations in vaginal fluid were significantly higher in pregnant women with pathologic findings on vaginal wet mount and spontaneous PTD [335-337]. This, however, contradicts with the findings of a study conducted by Kalinka *et al.,* (2005) where no significant difference in IL-1α, IL-1β, IL-6 and IL-8 concentrations in vaginal fluid of preterm versus term delivery groups were found [338].

Laskowska *et al.,* (2007) postulated the possibility of IL-8 and TNF-α having an active role in preeclamptic pregnancies with or without IUGR and reported a possible role of IL-8 in sequelae of preeclamptic pregnancies complicated by IUGR [339]. Another study found high levels of IFN-γ and increased production of TNF-α in peripheral blood

of pregnant women with recurrent pregnancy loss (RPL) [340]. Consequently Talukdar *et al.,* (2008)concluded that increased inflammatory cytokine IFN-y and IL-17-producing gamma T cells were associated with generating inflammatory cytokines that could contribute to loss of pregnancy in women with recurrent pregnancy loss compared to normal [341].

1.2.17 Cytokines as biomarkers for adverse birth outcomes

As most women with BV and many infections that lead to adverse birth outcomes lack clinical signs or symptoms, a substantial amount of research has focused on the identification of potential biomarkers to predict PTD, although few have proven sufficiently reliable or accurate for prediction of PTD [342]. However, bedside tests that would offer quick and timely clinical results for treatment studies are being developed to identify potential biomarkers for diagnosis for intraamniotic inflammation in preterm labour [343, 344]. Amniotic fluid (AF) point of care (POC) tests were evaluated against enzyme-linked immunosorbent assay (ELISA) measuring IL-6 concentration, a key cytokine for intra-amniotic inflammation in women who presented with preterm labour [344]. Interleukin 6 was chosen since it has been used as an excellent method for detection of intra-amniotic inflammation. Women in preterm labour would have increased concentrations of IL-6 and this could be ideal for evaluating the predictive values of the POC test [343, 344]. The ability of the AF IL-6 POC to identify microbial invasion of the amniotic cavity was comparable to ELISA with a high specificity and sensitivity of 91% and 93% respectively for correctly assaying IL-6 levels. Furthermore, another POC test (AF IL-6 and IP-10) was evaluated which included Interferon-γ induced protein (IP-10) and IL-6 and was found to strongly correlate with ELISA results [343].

A large proportion of these studies have focused on biomarkers in the amniotic fluid [345] which could be useful after rupture of membranes, but it would not be feasible to perform amniocentesis routinely to screen women who are not in labour for PTD risk [346]. In non-pregnant South African women, it has been show that inflammatory cytokines (IL-1α, IL-1β and IP-10) measured in vaginal swab samples may serve as useful biomarkers to identify women with asymptomatic bacterial/protozoan STIs or BV [220, 347, 348]. A similar tool may prove useful for identifying pregnant women with asymptomatic dysbiosis and STIs who are at risk of adverse birth outcomes.

1.2.18 Microbiome Analysis

The examination of DNA to study microbial communities is termed *metagenomics*. Metagenomics has two major approaches: shotgun sequencing, which randomly sequences all genomic DNA in a sample revealing the gene and taxonomic content [114] and amplicon sequencing, where only a selected part of the genome is sequenced, for example, the 16S rRNA gene. Metagenomics allows for characterization of relative abundances of microbes. It further allows characterization of the taxonomic diversity within samples, called alpha diversity, and between communities, called beta diversity [349] (**Figure 1.9**). Currently all studies investigating microbial communities employ high throughput sequencing and may also employ metabolomics and metaproteomics approaches [350]. In this study 16S rRNA is used for vaginal microbiota analysis it identifies a large number of vaginal bacterial, 16S rRNA identify microorganisms with a degree of accuracy as the highly conserved region are unique to genera, species and strains. It helps eliminate human and viral DNA, furthermore, the microbial genome is relatively smaller that it is cost effective [351]. However, it has some limitations in that it sequences only a single region of the bacterial genome and has a short length read, it has limited resolution and sensitivity on assigning taxonomic affiliation for organism identification when compared to shotgun sequencing. Shotgun sequences has advantage in that n it provides enhanced detection of bacterial species, increased detection of diversity and increased prediction of genes and also, improved the accuracy of species detection [351] [352].

1.2.19.1 *16S rRNA gene sequencing*

For studying bacterial taxonomy and profiling, identification of a conserved gene common to all bacteria that is also variable and differs between individual genomes is imperative [353]. The 16S ribosomal ribonucleic acid gene (16S rRNA), which is a gene encoding the RNA component of the 30S subunit of the bacterial ribosome, is often used. This gene possesses adequate taxonomic coverage, and is significantly ubiquitous with highly conserved flanking bases of nine hypervariable regions (V1-V9) [354]. Targeted amplicon sequencing of the 16S rRNA gene (1.5 Kbp) is used for determining the taxonomic composition and community diversity of a given sample. The most commonly used hypervariable regions are V1-V6 [355].

Selected genes are amplified using target specific primers that amplify conserved regions flanking the hypervariable regions. The samples are barcoded, then barcoded samples are pooled and purified using AMPure XP beads ((Beckman Coulter, Brea, CA, USA). Quantification is done using picoGreen double stranded DNA (dsDNA) assay (Invitrogen, Carlsbad, CA, USA). Dual indices and Illumina sequencing adapters are attached by using the Nextera XT DNA Prep kit (Illumina). Samples are again purified and then purified libraries consisting of _96 pooled samples are paired-end sequenced on an Illumina MiSeq platform (300-bp paired-end reads with V3 chemistry) [134, 356]. Raw reads generated from microbiome sequences are quality controlled, removing ambiguous reads and chimeras, and demultiplexed (assigned to samples using barcodes) [114]. In some pipelines, such as QIIME, raw reads are then binned into clusters of closely identical genomes called operational taxonomic units (OTUs) according to sequence similarity accommodating divergence of between 95-99% [356] **(Figure 1.9)**. However, newer methods include DADA2 (Divisive Amplicon Denoising Algorithm2) which yields amplicon sequence variant table (ASV) table (McMurdie and Holmes, Plos Comp Biol). Alignment and inference to phylogenetic trees is done using alignment programmes such as Fasttree, MUSCLE or PyNAST332 [357]. The final identification of the microbial data is performed by comparing it to gene databases that contain millions of referral sequences, such as the Ribosomal Data Base project (RDP), CAMERA, Green Genes, VAMPS, MG-RAST [358, 359] and SILVA. This assigns taxonomic lineages to each OTU [350]. OTUs are often identified down to the Family and sometimes Genus or species level. The Illumina MiSeq is an integrated instrument that performs clonal amplification, genomic DNA sequencing, and data analysis with base calling, alignment, variant calling, and reporting in a single run. The Illumina

Miseq platform is most commonly used for amplicon sequencing, but due to the limited read lengths of ~400bp (excluding primers and adapters), often species level annotation is difficult.

Mothur [356] and Quantitative Insights Into Microbial Ecology (QIIME) are Bioinformatics tools commonly used to filter and analyse raw reads. Analysis with QIIME results in quality filtered OTUs generated using UCLUST, which can produce taxonomic tables, phylogenetic trees and visual diversity plots [360] **(Figure 1.9)**. OTUs are presented as summarized data tabulated into abundances and annotated into taxa across samples, for downstream statistical analysis [21]. The tabulated data can be stored in a Biological Observation Matrix (BIOM) file for ease of use within different bioinformatics tools [21].

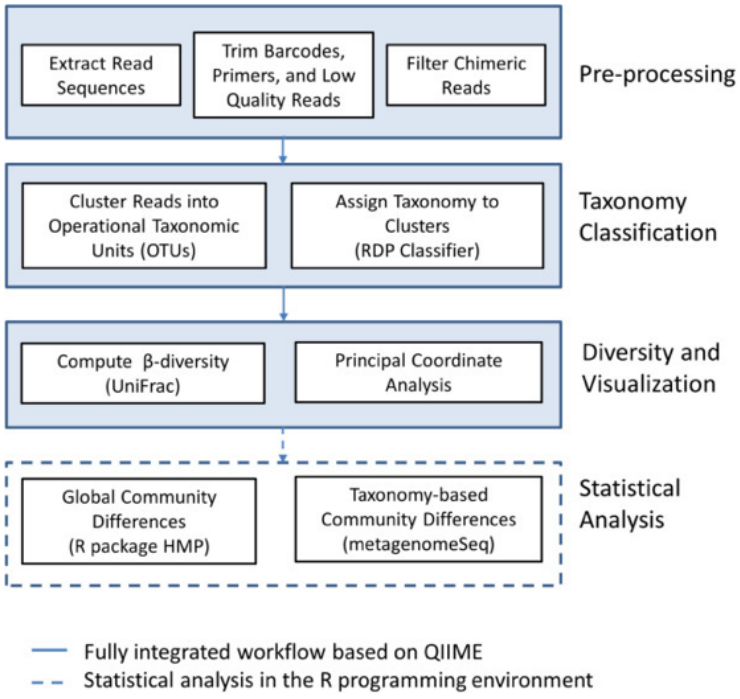


Figure 1. 9 QIIME based analysis

QIIME-based analysis is performed in three steps: (1) pre-processing, (2) taxonomy classification, and (3) computation of diversity and visualization. Further statistical analysis of the data is performed in R programming environment (Jun Hang *et al.,* 2014)

1.2.20.2 *Statistical analysis of microbiome data*

Statistical approaches are important to assess diversity of microbial communities and their dynamic interaction with the environment and the host. The main challenges with microbial data include data sparcity (i.e. zero inflation), compositionality and sequence depth and distribution, which is not always comparable between samples. Nonparametric methods are used for analysis for data which is not normally distributed [21]. Rescaling and rarefying of reads has been employed in order to try and transform data to suit models to be used by different algorithms such as metagenomSeq, edgeR [361] and DESeq [362, 363]. Open access software such as Calypso [364] and STAMP [365] incorporate these tools. These software models focus on multivariate statistical approaches and permit quantitative visualization of microbial communities.

Statistical analyses performed on microbiome data include comparisons of alpha and beta diversity metrics. Diversity within a sample that takes into account evenness (relative abundance of species), and richness (total number of species present) is called alpha diversity. The commonly used metrics are Chao1, Simpson, and Shannon indices [357]. Dissimilarity between each pair of samples or environmental gradients, characterised by the number of species shared between the communities, is called beta diversity. The commonly used beta diversity metrics are Bray-Curtis distances, unweighted and weighted UniFrac distances, depending on which is suitable to the parameter in question. Non-metric multidimensional scaling (NMDS) or principal coordinate analysis (PCoA) are commonly used for visualization of ordination data [21, 357].

Hierarchical clustering and heat maps are used to visualize the composition of communities [21]. LEfSe (Linear discriminant analysis Effect Size) has been used to determine biomarkers for relevant features of interest [366], while Random Forest is a combination tree predictor which depends on independently sampled random vectors [367]. Analyses used to assess statistical significance can employ nonparametric tests such as Kruskal-Wallis rank sum or Mann Whitney U test to determine groups or abundances with statistical differences [357].

1.3 Research hypothesis and objectives

Hypothesis

- There is an association between the vaginal microbiota and adverse pregnancy outcomes.

Aim

- To characterise the vaginal microbiota in relation to pregnancy outcomes.

Specific objectives

1. To determine the association between the vaginal microbiota and pregnancy outcomes.
2. To compare the vaginal microbiota in pregnant women with or without HIV.
3. To estimate the prevalence of BV and other vaginal pathogens in pregnant women.
4. To determine the immunological and microbiological biomarkers or predictors of adverse pregnancy outcomes

Chapter 2: Methodology and Study Design

2.1 Study cohort

This was a prospective observational cohort study of pregnant women who attended the antenatal clinics (ANC) at Harare and Chitungwiza central hospitals. The study was carried out in two central hospitals providing antenatal services for high density suburbs and for referral cases from peri-urban clinics and rural district hospitals.

Inclusion criteria: All pregnant women were 13-35 weeks of gestation, 18 years of age and above, willing to provide written consent and participate in the study. Additionally, all women were not receiving treatment for any STI (except HIV) nor received antibiotic treatment in the past one month with documented HIV testing results in pregnancy.

2.2 Ethical considerations

This study obtained approvals from all of the institutions involved. Harare Central Hospital (HCHEC 080416/21) and Chitungwiza Central Hospital ethics committees (dated 28/04/16) approved sample collection at their hospital antenatal clinics. The study was approved by the University of Cape Town Human Research Ethics Committee (HREC/REF 380/2016) and Medical Research Council of Zimbabwe (MRCZ A/2054). The Research Council of Zimbabwe (RCZ) also approved shipment of samples to University of Cape Town, South Africa.

2.2.1 Consenting Processing

The consenting procedure was done by qualified trained research nurses. The study, including aims, was explained in the language of choice to all pregnant mothers during the educational sessions. To ensure understanding of the study and the collection procedures, study participants were asked to repeat back their understanding, and signed consent for storage of specimens and shipping for further testing in South Africa.

2.3 Study procedures

At enrollment, socio-demographics, reproductive health history, antiretroviral use, CD4 count and previous pregnancy outcomes were collected using a questionnaire. Vaginal samples were collected from consenting participants and labelled with a study code, e.g. BVDZIV001. The research nurses and assistants stationed at each clinic collected patient background data as indicated on the questionnaire, including ultimately, pregnancy outcomes.

2.3.1 Sample size calculation

Sample size was calculated using a single proportion estimate (BV prevalence) with specified precision. It was found that a sample size of 340 would provide 80% power to detect the magnitude of difference in BV prevalence between HIV infected and uninfected pregnant women (alpha=0.05) [28].

Sample size to estimate a single proportion	
Estimated Proportion	0.33
Confidence level	0.95
Desired precision of estimate	0.05
Population size	unknown
Results	
	Sample size
Infinite population	**340**

The estimated sample size become approximately 340 + 5% attrition gives a total minimum sample size of **357**

2.3.2 Vaginal swab collection procedure and storage

Two vaginal swabs were collected from each pregnant woman during their antenatal visit checkup at enrollment . The research nurses collected specimens aseptically by inserting the swab 2cm into the vagina, swabbing inside the vaginal canal by rotating the swabs for approximately 5 seconds. Upon removal of the first swab, it was touched on the pH paper and bedside pH was recorded. The swab was immediately placed into the transport tube with 1.5mL sterile phosphate buffered saline (PBS), placed in the cooler box with ice packs (2-8^{0}C) and transferred to the laboratory within 4 hours of collection where it was stored in a -70^{0}C freezer and later shipped on dry ice to South Africa. A second vaginal swab was sent to the laboratory used for culture and

microscopy. All women were tested for syphilis and HIV during their first antenatal visit.

2.4 Laboratory diagnosis

2.4.1 Microscopy and culture

Nugent score and Hay /Ison technique (gram stain)

Diagnosis of bacterial vaginosis was done by Gram stain and microscopy by experienced staff using the Nugent scores and Hay Ison technique **(Table1.1 & Table1.2)**. Swab samples were smeared onto glass slides and stained with Gram statin reagents. Women were grouped at BV negative (predominantly lactobacilli; score: 0-3), BV intermediate (some lactobacilli and some gram variable coccobacilli and *Mobiluncus*; score: 4-6) or BV positive (few to no lactobacilli with abundant gram variable coccobacilli and *Mobiluncus*; 7-10) based on scoring conducted by three laboratory technicians. A wet prep was done for *Trichomonas vaginalis* diagnosis.

Culture

Swabs in Amies transport media were cultured on Columbia Blood agar (CBA) with 5% sheep blood, MacConkey (MAC) agar plate (Oxoid, UK). These were incubated in anaerobic jars at 37°C for 18-48 hours and MAC was incubated aerobically at 37°C for 18-48 hours. Gram positive cocci were sub cultured on Columbia colistin and nalidixic acid (CNA) agar and chromogenic agar (ChromID Strepto *B agar*; STRB; bioMérieux, Inc) for group B streptococcus. Biochemical tests were performed on suspected pathogenic organisms using sub-cultured pure fresh colonies of 0.5 McFarland standard equivalent suspension. Species level identification for gram negative organisms was done using API 20E (Biomerieux, UK). Suspected group B streptococcus was identified using the Christie, Atkins, and Munch-Peterson (CAMP) test and the serologically using Rapid latex agglutination test Streptex (ThermoFischer scientific.US), for quality control throughout culture methods the procedure *Escherichia coli* ATCC 25922 and *Staphylococcus aureas* 25923 were used Matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF) was used for confirmation of identified GBS isolates

2.5 Microbiome analysis

2.5.1 DNA extraction

DNA extraction was done using an inhouse method as published by Anahtar 2016. Optimization was done using aliquots of stored vaginal samples and the yields quantitated by gel electrophoresis and Qubit. It was found that phenol chloroform extraction method yielded a considerably greater amount of DNA compared to commercial Zymol kit. After this optimization study, samples were then processed.

Vaginal swabs were thawed, scraped of vaginal fluid for maximum yields and 500µl was transferred to a sterile Eppendorf tube. To obtain lysis of gram-positive bacteria a cocktail of enzymes including 3µl lysostaphin (4kU, Sigma Aldrich), 6µl mutanolysin (25kU/ml, Sigma Aldrich) and 50µl lysozyme (450kU/ml, Sigma Aldrich) were added to the samples [368]. The mixture was incubated for 1hr at 37^0C, vortexing every 10-20 minutes. After enzymatic digestion Sodium deodecyl sulfate (SDS), Phenol:chloroform:isoamyl alcohol and prepared buffer were added to the vaginal fluid mixture and then it was subjected to bead beating. DNA extraction was done using the phenol chloroform extraction method [369] (URL: http://www.jove.com/video/53939). Isopropanol precipitation and ethanol wash was done and the nucleic acid pellet was resuspended in ultra-pure Tris EDTA buffer and DNA concentration was quantified using the Qubit dsDNA high sensitivity reagent (Invitrogen). DNA was run on a 1.5% agarose gel for visualisation. The extracted DNA was stored at -80^0C for library preparation.

2.5.2 Library preparation

The V4 hypervariable region was selected in this study because it allows a better discrimination of BV-associated bacteria. The hypervariable V4 region of the 16S rRNA gene was amplified by nested PCR using universal primers (515F/806R).(Table 2.1) [370]. PCR1 specific primers with overhang adapters were used to amplify the V4 template out of the DNA sample. Due to general high yields of DNA, samples were not processed in duplicate, but template was increased per sample during the first PCR to approximate 20ng DNA template. The first PCR conditions were as follows: 95°C for 3 minutes, then 35 cycles of: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 30 seconds, then termination at 72°C for 5 minutes. Purification of the 16S rRNA gene V4 amplicon to remove free primers and

primer dimers was done using AMPure XP beads (Beckman Coulter, Brea, CA, USA). Quality control was done using Qubit for quantification and agarose gel electrophoresis for visualisation of bands.

The purified amplicons were subjected to 2nd PCR: The Nextera XT Index Kit (Illumina) was used during this stage where engineered transposomes simultaneously fragment and tag ("tagment") input DNA adding unique sequencing adapters. These adapters are used during the PCR reaction to amplify the insert DNA. Index sequences are added on both ends of the DNA, enabling dual-indexing of pooled libraries. The reaction contained a total volume of 50µl with 5µl DNA amplicon from the 1ST PCR. The reaction started at 95°C for 3 minutes and went through 8 cycles at the same temperatures as in PCR 1 above. Quality control was done using the Qubit fluorometer (Invitrogen) for quantification and verification was done by gel electrophoresis for visualisation of bands. Samples were pooled in equimolar amounts and the pooled library was run on an agarose gel (1.5 %). Purified libraries consisting of 96 pooled samples per library were stored in -20°C for shipment to Seattle USA for sequencing using Illumina MiSeq platform.

2.5.3 Sequencing

To quantify each library's amount of adapter-ligated DNA a combination of NEBNext Ilumina Quantification Kit and a Qubit dsDNA Broad Range assay were used. Libraries were sequenced from both ends using the 300-bp paired-end kit with v3 chemistry (MiSeq v3 2x300bp kit) and the resulting reads were demultiplexed on the MiSeq.

Table 2. 1 16S rDNA V4 Amplicon Library Primers

Primer	Primer sequence (5' to 3')
Primary PCR primers	
Modified 515F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA
Modified 806R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT
Secondary PCR primers	
501	AATGATACGGCGACCACCGAGATCTACAC**TAGATCGC**TCGTCGGCAGCGTC
502	AATGATACGGCGACCACCGAGATCTACAC**CTCTCTAT**TCGTCGGCAGCGTC
503	AATGATACGGCGACCACCGAGATCTACAC**TATCCTCT**TCGTCGGCAGCGTC
504	AATGATACGGCGACCACCGAGATCTACAC**AGAGTAGA**TCGTCGGCAGCGTC
505	AATGATACGGCGACCACCGAGATCTACAC**GTAAGGAG**TCGTCGGCAGCGTC
506	AATGATACGGCGACCACCGAGATCTACAC**ACTGCATA**TCGTCGGCAGCGTC
507	AATGATACGGCGACCACCGAGATCTACAC**AAGGAGTA**TCGTCGGCAGCGTC
508	AATGATACGGCGACCACCGAGATCTACAC**CTAAGCCT**TCGTCGGCAGCGTC
701	CAAGCAGAAGACGGCATACGAGAT**TCGCCTTA**GTCTCGTGGGCTCGG
702	CAAGCAGAAGACGGCATACGAGAT**CTAGTACG**GTCTCGTGGGCTCGG
703	CAAGCAGAAGACGGCATACGAGAT**TTCTGCCT**GTCTCGTGGGCTCGG
704	CAAGCAGAAGACGGCATACGAGAT**GCTCAGGA**GTCTCGTGGGCTCGG
705	CAAGCAGAAGACGGCATACGAGAT**AGGAGTCC**GTCTCGTGGGCTCGG
706	CAAGCAGAAGACGGCATACGAGAT**CATGCCTA**GTCTCGTGGGCTCGG
707	CAAGCAGAAGACGGCATACGAGAT**GTAGAGAG**GTCTCGTGGGCTCGG
708	CAAGCAGAAGACGGCATACGAGAT**CCTCTCTG**GTCTCGTGGGCTCGG
709	CAAGCAGAAGACGGCATACGAGAT**AGCGTAGC**GTCTCGTGGGCTCGG
710	CAAGCAGAAGACGGCATACGAGAT**CAGCCTCG**GTCTCGTGGGCTCGG
711	CAAGCAGAAGACGGCATACGAGAT**TGCCTCTT**GTCTCGTGGGCTCGG
712	CAAGCAGAAGACGGCATACGAGAT**TCCTCTAC**GTCTCGTGGGCTCGG

The adapter sequences are underlined in the 501 and 701 secondary primer sequences. The same sequences are found in each of the 500 and 700 primer series. The 8-nucleotide sample indices are bolded.

2.5.4 Microbiome data analysis

16S rRNA gene amplicon sequencing analysis was performed as previously described by Lennard et el. (2018). Briefly, after demultiplexing, raw reads were preprocessed as follows: forward and reverse reads were merged by using usearch7 [371] allowing a maximum of three mismatches; merged reads were quality filtered by using usearch7 (reads with E scores of >0.1 were discarded); primer sequences were removed by using a custom python script; and merged, filtered reads were truncated at 250 bp. Dereplicated sequences were sorted by abundance (highest to lowest) and clustered *de novo* into operational taxonomic units (OTUs) at 97% similarity using usearch7. Chimeric sequences were detected (against the Gold database) by using UCHIME [372] and removed. Individual sequences were assigned to the specific identifiers using a 97% similarity threshold. Taxonomic assignment was performed with QIIME 1.8.0 [355] using the RDP classifier (using the default confidence level of 0.5) against the Greengenes 13.8 reference taxonomy for 97% identity. Down stream annotation to species level for few key taxa was performed using both NCBI and Lennard *et al.,* (2018) databases (Supplemental material found at https://doi.org/10.1128/IAI.00410-17). Lennard *et al.,* (2018) compared green genes assigned taxonomy,16S vaginal reference database taxonomy (updated from Fettweis *et al.,*2012) and BLAST against NCBI nucleotide database and constructed a consensus taxonomy for downstream analyses [134]

Samples with ≥ 5,000 reads were selected for downstream analyses. The OTU table was standardized (i.e., transformed to relative abundance and multiplied by the median sample read depth) and filtered so that each OTU had at least 10 counts in at least 2% of samples or a relative abundance of at least 0.001%.

2.5.5 Downstream statistical analysis

Downstream analysis was done in R. The *phyloseq* package, which provides a set of classes and tools to facilitate the import, storage, analysis, and graphical display of microbiome census data, was used which (downloaded from Bioconductor "http://www.bioconductor.org/biocLite.R"). Alpha (α) diversity estimates (Shannon, Simpson) and richness (Chao1) were calculated using the R package vegan [373]. Beta-diversity was estimated by NMDS of Bray-Curtis distances matrices or PCoA [21]. The package ggplot2 was used to build various graphs from the same set of data.

Annotated heat maps were constructed using package NMF [374] on merged taxa at lowest taxonomic levels for visualization. Random forest analysis [367] was used to determine taxa most predictive of different conditions while differential abundance testing was done using metagenomeSeq [363]. A Bray distance object with silhouette-optimal number of clusters was generated using the vegdist function which computes good rank order community dissimilarity indices, and fuzzy clustering produced the three community state types (CST1-CST3) named according to the dominant species in the clusters. For calculating relative risk and odds ratio epitools, tidyverse and other packages nested in the custom function R script were used [134]Logistic, multivariate and multinomial regressions were performed in R using the lm(), glm() and mlogit() functions respectively. MetagenomSeq was used for differential testing of microbiota between groups where these OTUs were defined as differentially abundant as they met the threshold criteria of FDR adjusted p= 0.05 absolute FC 1.25 and percentage presence in at least one group of 20%. LEfSe (Linear discriminant analysis effect size) is a tool used to find biomarkers between 2 or more groups using relative abundances [375]

2.6 Cytokine analysis

Cytokines were measured to assess biomarkers for BV as well as predictors of adverse birth outcomes. Concentrations of 27 cytokines (FGF basic, Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF) were measured in vaginal fluid using Luminex (R&D).

2.6.1 Measurement cytokines in vaginal fluid by Luminex

Samples were thawed overnight at 4°C, the liquid phase separated from the solid phase through centrifugation at 13000 rpm for 10 minutes, and this liquid fraction filtered using a Spin-X tube (Corning Costar, 0.22um filter) at 13000 rpm for 10 minutes. Concentration of 27 cytokines were measured from the filtrate using a Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad Laboratories Inc., USA). Assay plates were read using a Bio-Plex Suspension Array Reader (Bio-Rad Laboratories Inc., USA). Data were analyzed using Bio-Plex manager software (version 4). Cytokine levels that were below the lower limit of detection of the assay were reported as the mid-point between zero and the lowest detectable level measured for that given cytokine. As

samples were spread across three plates, a reference panel of five samples was included on each of the plates (inter-plate controls), in addition to five samples being duplicated on each set of plates (intra-plate controls) for quality control measures. Spearman's rank to measure intra-assay and inter-assay correlation coefficients was used to determine assay reliability and reproducibility **(Appendix A).**

2.6.2 Statistical analysis

Shapiro-Wilk test for normality was performed to determine the distribution of variables within the dataset. Comparison of unpaired non-parametric data was done using the Mann-Whitney U test. The Spearman's rank test was applied to test for correlation between nonparametric data. Statistical inferences on binary sets of data were performed using the Fisher's exact test and odds ratios calculated. Non-parametric assessments of variation between groups was carried out through Chi-square test and the Kruskal-Wallis Analysis of Variance (ANOVA), with Dunn's post-test being applied to test for the effect of multiple comparisons. Statistical analyses were performed using mainly R software, and also GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, USA). All tests were two-tailed and p-values of ≤ 0.05 after adjusting for multiple comparisons were considered significant. Confirmatory factor analysis was used to group cytokines together and generate factor scores representing the overall level of inflammation in each woman. Women were grouped as having high inflammation (upper quartile; $\geq 75^{th}$ percentile), medium inflammation (interquartile; $<75^{th}$ - $>25^{th}$ percentile) and low inflammation (lower quartile; $<25^{th}$ percentile).

Chapter 3: Results

3.1 Cohort characteristics

Four hundred and twenty-one women consented to participate in the study. One was excluded after she was later found to be ineligible due to gestational age. Socio-demographic characteristics such as marital status, level of education, smoking, alcohol intake, vaginal douching and referral reason, can be found in Table 3.1. There were more pregnant women recruited during their third trimester (63%; 265/420) compared to the 2nd trimester (37%; 155/420); **Table 3.1**). Five women were positive for syphilis (1.2%; 5/420), there were 48 (11.4%) HIV-infected women. The median gestational age at recruitment was 30 weeks while the median maternal age was 29.5 years. Surprisingly, most of the women were married (98%, 412/421), however there could be a potential information bias as this was self-reported data and could have been influenced by common stigma in the African society associated with being pregnant out of wedlock. Half of the women reported practicing vaginal douching (50.5%; 212/420) during pregnancy. Fifty-one (12.1%; 51/420) had used antibiotics in the past 3 months and of these, 10 were cotrimoxazole prophylaxis. Almost half of the women were referred due to poor obstetric history (49%; 206/420) in their prior pregnancies, which also included previous stillbirth, preterm birth and preterm labour (13%; 56/420, 13%; 56/420, 16.8%; 71/420, respectively), among others. Other reasons for referral included conditions during this pregnancy such as anaemia (n=11), pregnancy induced hypertension (n=16), antepartum haemorrhage (n=5), multiple pregnancy (n=7), prior pregnancy complications (n=5), cervical insufficiency (n=5) and elderly primip (n=1). Bacterial vaginosis (BV) by microscopy was present in 99 (23.5%) women, intermediate scores 63 (15%), BV negative 258 (61.4%) and only 14 (3%) had *Trichomonas vaginalis* **(Table 3.1).** Women in this cohort were asymptomatic for BV.

Table 3. 1 Clinical characteristics of the cohort

	Sample size N=420	%
Trimester: 2nd (13-27 weeks)	155	36.9
3rd (28-40 weeks)	265	63.1
HIV Positive	48	11.4
HAART Regimen (n=48) n (%):		
TDF, FTC, EFV	42	(89.1)
Combivir (AZT+3TC)	4	(8.3)
Nugent score: BV (7-10)	99	23.5
Intermediate (4-6)	63	15.0
Normal (0-3)	258	61.4
T. vaginalis	14	3.0
Antibiotic use past 3 months*	51	12.1
Syphilis Positive	5	1.2
Smoking partner	36	8.6
Level of education: Tertiary	17	4.0
Secondary	384	91.4
Primary	7	1.7
Referrals with condition	206	49.0
Non-referrals	214	51.0
History Stillbirth	56	13.0
History Preterm	56	13.0
Previous preterm labour	71	16.9
All previous poor outcomes	111	26.4
Vaginal canal opening	37	8.8
Vaginal douching	212	50.5
Parity: Nulliparous	112	26.6
Parous	308	73.3
Marital status: Married	412	98.0
Single	7	1.7
Divorced	1	0.2
	Median	**Range /SD**
Median maternal age	29.5	(24.6-35.3)
Median gestational age at recruitment, weeks	30	(27-32)
Blood CD4+ [cell/mm^3(n=24)] [median (IQR)]	495	(398.8, 643.5)
Plasma viral load copies/ml (n=8) (mean (SD)	128.85	(98.49)

* *10 were using cotrimoxazole prophylaxis; SD: Standard deviation*

3.2 Vaginal microbiota bacterial composition

3.2.1 Sequencing

Three hundred and ninety nine vaginal swabs from pregnant women were sequenced. Women with *T. vaginalis* (n=14) or Diabetes Mellitus (DM; n=7) were excluded to reduce bias, as *T. vaginalis* and DM are highly associated with poor reproductive outcomes. DNA was quantified by using the Pico Green double-stranded DNA (dsDNA) assay (Invitrogen, Carlsbad, CA, USA) as elaborated in methodology. A total of 32 samples did not yield enough DNA on amplification as evidenced by lack of visible bands on gel electrophoresis after PCR 1, leading to sequencing of 367 samples. The total sequencing reads were 27,591,809 (range 61 reads to 352 921 as observed on the rarefaction curve to check quality) and furthermore, we considered samples which passed quality control with 5,000 or greater reads for downstream analyses, resulting in 356 samples and 390 taxa retained. Three phyla with highest number of reads were Firmicutes, Actinobacteria and Bacteroidetes in that order **(Figure 3.1)**. Greengenes was used to assign OTUs to sequences and 1185 OTUs were generated as described in detail in Chapter 2. A phylogenetic tree with 1165 tips and 1163 internal nodes was constructed. After standardizing, we filtered samples selecting OTUs where the row sum for that OTU had at least 20% of samples with a count of 10 each or where that OTU > 0.001% of the total median count.

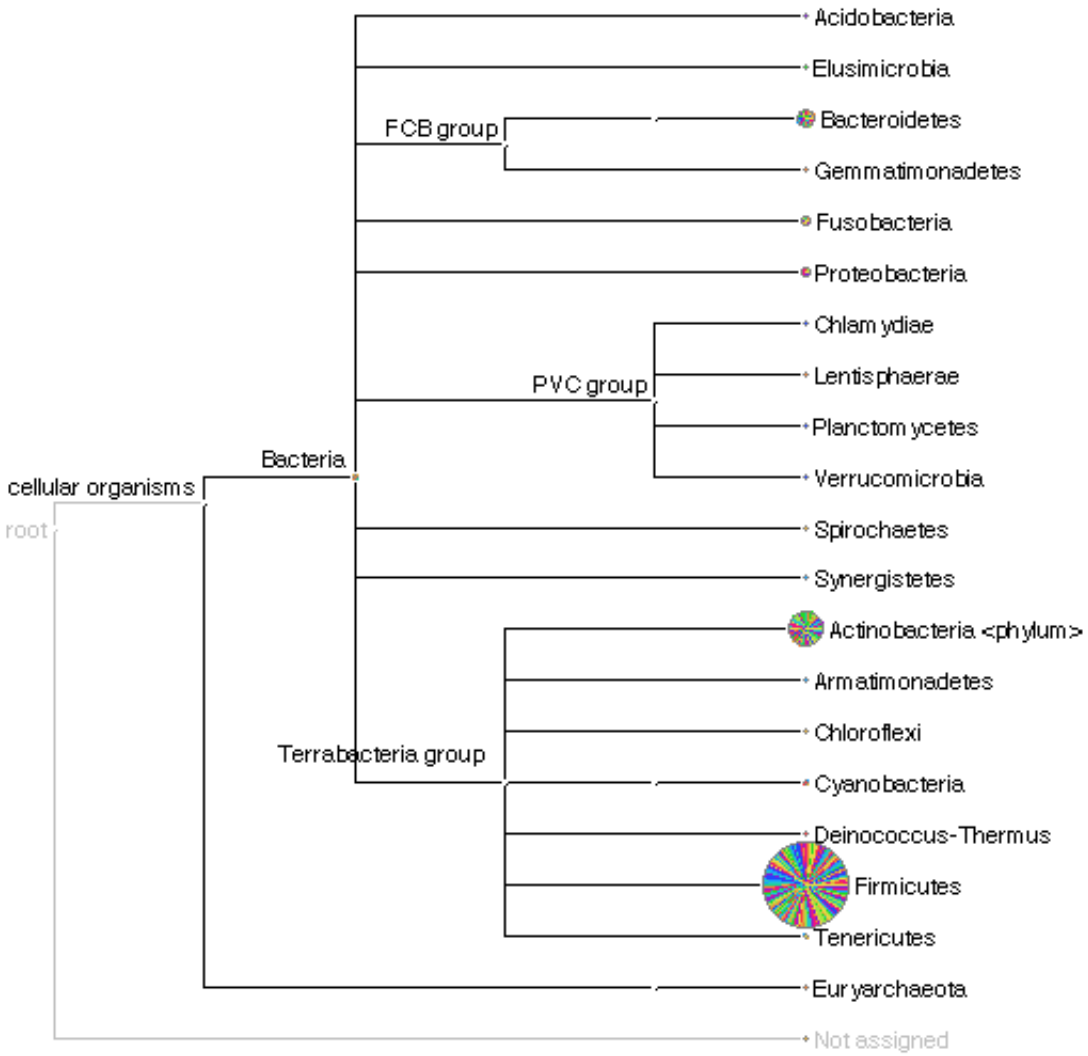


Figure 3.1 Taxonomy phylum level showing the proportion of reads per phylum. There were 20 phyla identified. The size of the circle is proportionate to the number of reads obtained from that particular phylum.

3.2 1.1 Annotating OTUs

Renaming of OTUs that were not annotated to species level was done through blasting the OTU in the NCBI database where possible **(Appendix B)**. The OTUs that were 100% identified were renamed as per NCBI and some key taxa were annotated according to Lennard *et al.* (2018) database **(Table 3.2)** (Supplemental material found at https://doi.org/10.1128/IAI.00410-17). They created a curated taxonomic database by using the Usearch_global command implemented in VSEARCH to search for representative sequences of *de novo*-picked OTUs against the updated custom vaginal 16S reference database, accepting all hits with ≥97% identity. Annotations which conflicted with the corresponding Greengenes annotations were BLAST searched against the NCBI nucleotide database (excluding uncultured organisms) for all OTUs with hits against the custom vaginal 16S reference database and the results were then manually curated [134].

Table 3.2 Taxa annotation

OTU	**Green genes**	**NCBI**	**Lennard *et al.*, 2018**
OTU_11	*Gardnerella*	*Gardnerella vaginalis*	*Gardnerella vaginalis*
OTU_ 11	*Lactobacillus*	*Lactobacillus crispatus & Lactobacillus acidophilus*	*Lactobacillus crispatus_acidophilus*
OTU_745	*Lactobacillus*	*Lactobacillus jensenii*,	*Lactobacillus jensenii*,
OTU_8	*Prevotella*	*Prevotella timonensis*	*Prevotella timonensis*
OTU_13	*Shuttleworthia*	*No good hits*	*BVAB1*
OTU_40	*Neisseria cinerea*	*N. gonorrhoeae*	*N. gonorrhoeae*
OTU_110	*Atopobium*	*Atopobium minitum*	*Atopobium minitum*
OTU_85	*Atopobium*	*Atopobium rimae*	*Atopobium rimae*

3.2.2 Relative abundance

The median relative abundance at phylum level revealed that Firmicutes and Actinobacteria were the most abundant phyla. **(Figure 3.2).** At genus level *Lactobacillus* and *Gardnerella* had a high mean relative abundance; their microbial content was higher than 1% median sample read depth showing their level of dominance in the vaginal microbiota of this cohort (**Figure 3.3**). *L. iners* was present in majority of the pregnant women (>90%) at varying degrees of abundance. This was also true for *Gardnerella vaginalis* and *L. crispatus,* which were found in > 70 % of the women.

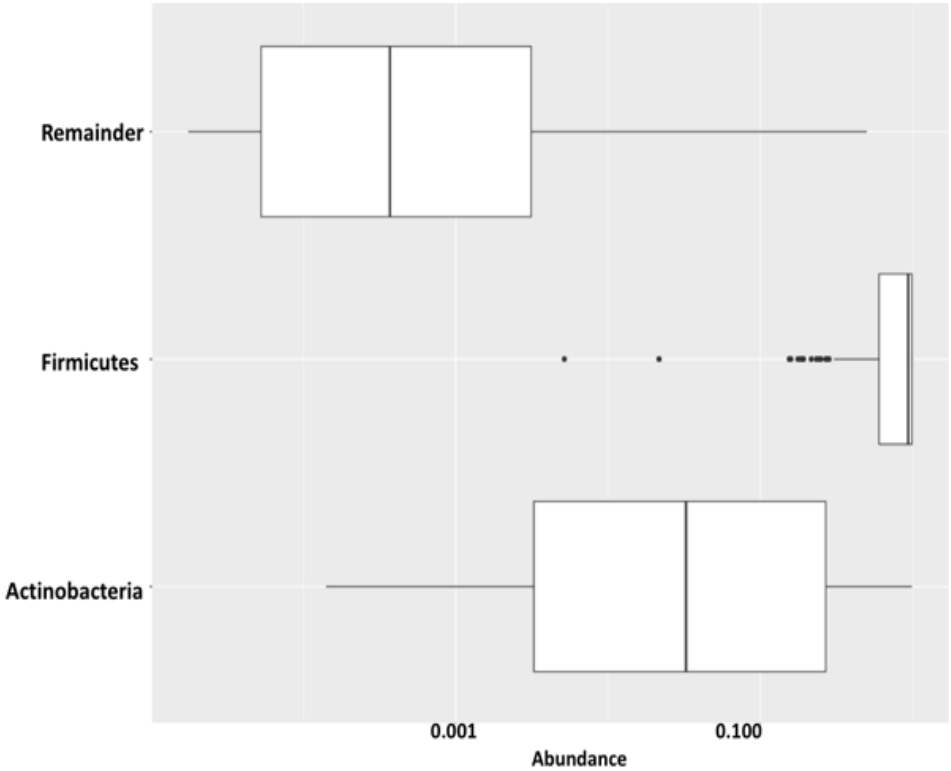


Figure 3. 2 Vaginal microbial community abundance in 356 pregnant women at phylum level. The relative abundance of phyla Firmicutes and Actinobacteria, which had > 0.01% median sample reads depth, is shown. Phyla with <0.01 were all grouped as Remainder .

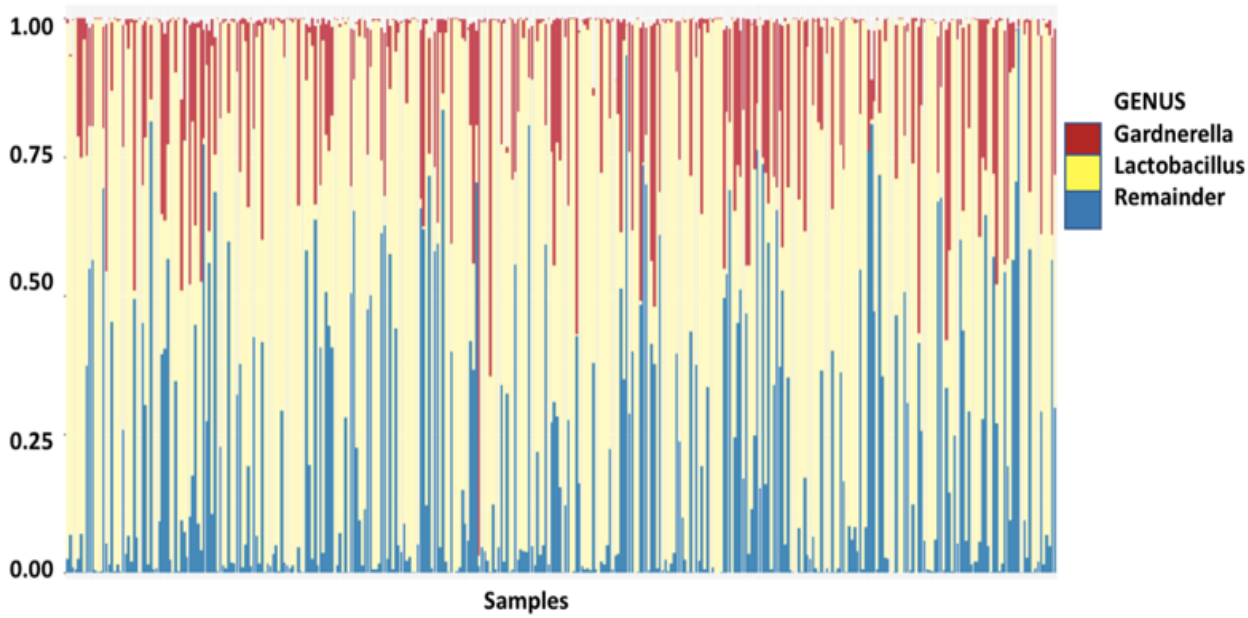


Figure 3. 3 Vaginal microbial community abundance in 356 pregnant women at genus level. The relative abundance of genera *Gardnerella* (red) and *Lactobacillus* (yellow), which had >0.01% median sample reads depth, is shown. Genera with <0.01 were grouped as Remainder and coloured in blue.

3.2.3 Vaginal community state types in pregnancy

The vaginal profiles of the pregnant women in this cohort clustered into three main CSTs using Fuzzy clustering of Bray Curtis distances. This was achieved by silhouetting the optimal number of clusters for the cohort which yielded three CSTs **(Figure 3.4).** Using the 16sRNA gene sequence count data, and the membership exponent of 1.25, with Bray Curtis as the dissimilarity measure used to form clusters, only members with a ≥60% probability of belonging to any of the three clusters were included in downstream analyses. The clusters were then named according to the dominant taxa in each optimal fuzzy cluster 1-3 as (CST1 - *Lactobacillus iners*, CST2 – *Lactobacillus crispatus*, CST3 - *Gardnerella vaginalis*) respectively (**Figure 3.5A**).

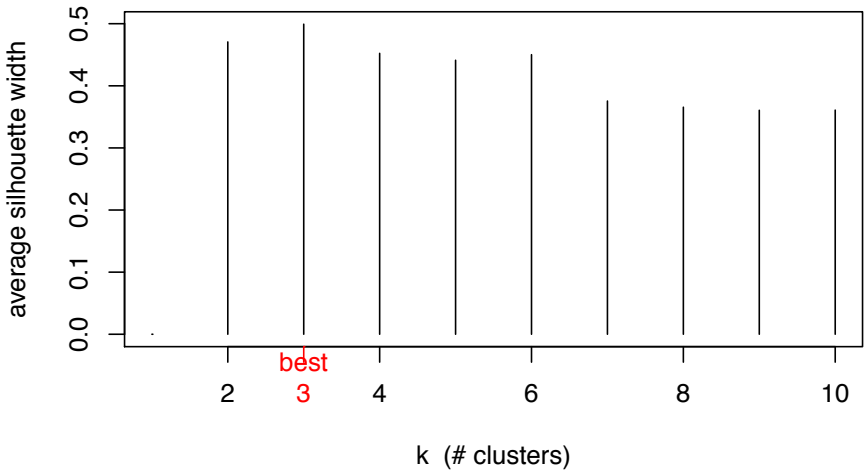
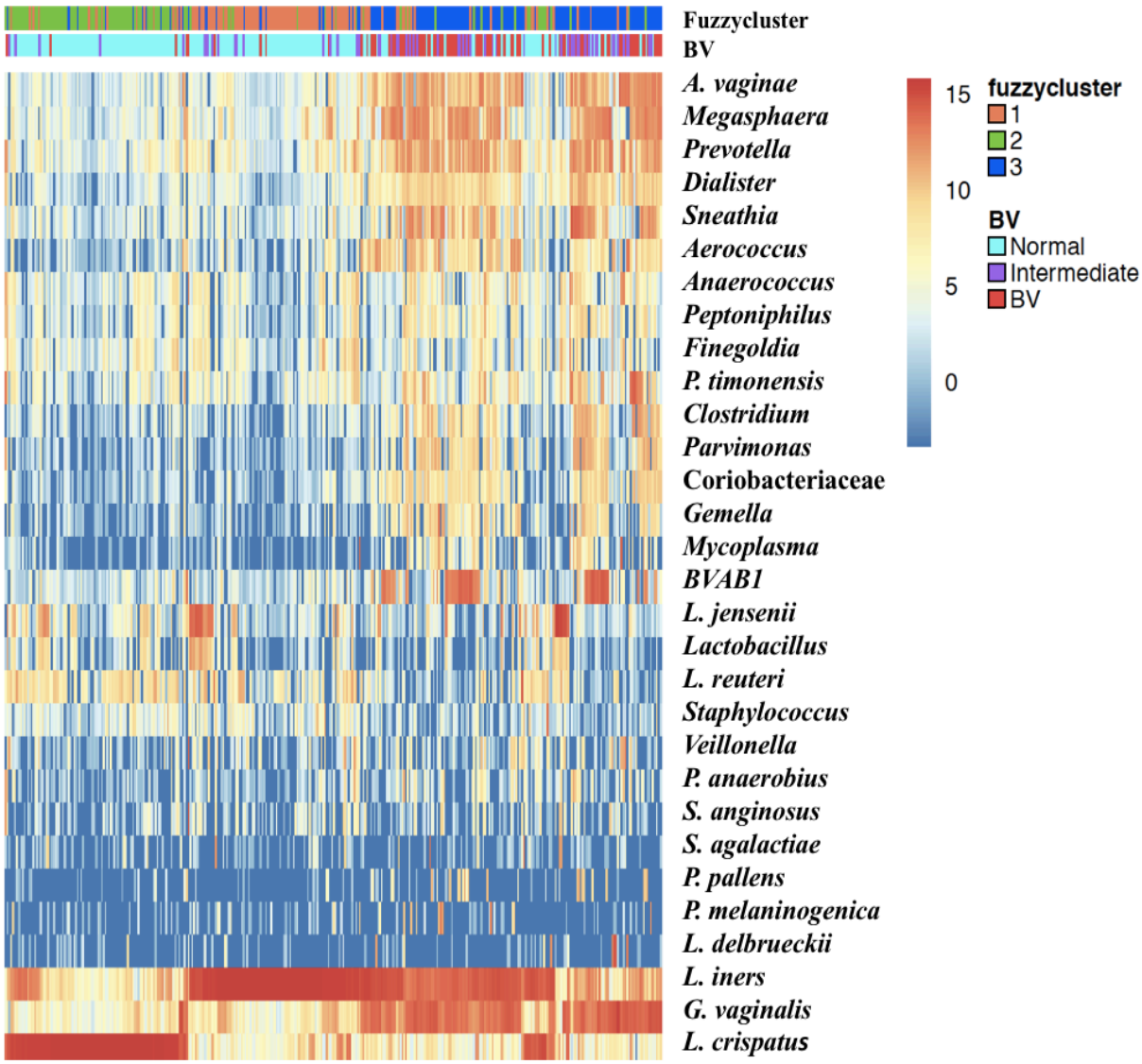


Figure 3. 4 Average silhouette width and optimal clusters. The optimal number of clusters (k) according fuzzy clustering of Bray Curtis distance is 3 as shown in red

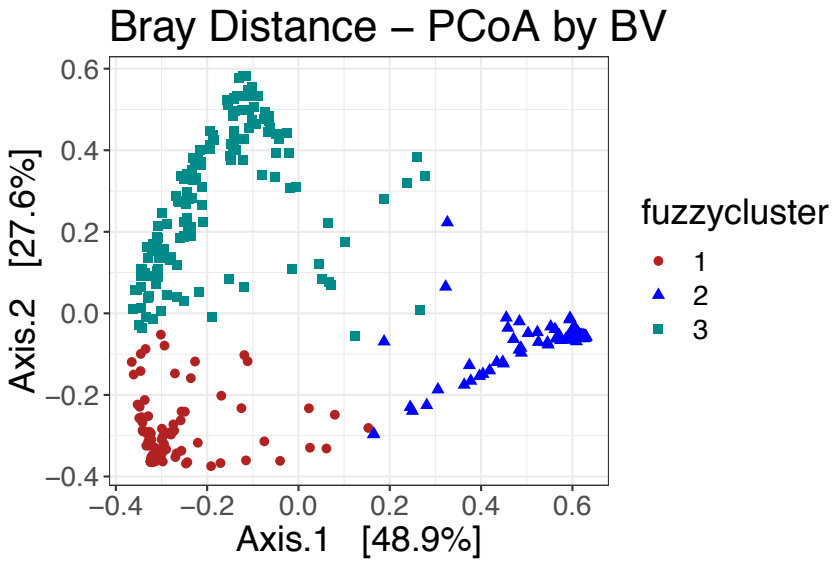
Unsupervised hierarchical clustering of merged taxa with greater than 1% abundance in at least one sample was performed using Bray Curtis distances. A heatmap was constructed using the top 30 most abundant taxa merged at the lowest taxonomic annotation **(Figure 3.5A).** Ordination using the PCoA method with Bray Curtis distance was performed which also revealed three clusters visually (**Figure 3.5B & C)**. CST3 (*G. vaginalis*-dominant) was a highly diverse community composed of a diverse array of strict anaerobic or facultative bacteria. These bacteria represented nearly >50% of the most abundant taxa which included *Prevotella, Dialister, A. vaginae, Megasphaera, Sneathia, Aerococcus, Gardnerella, Clostridium, Parvimonas, Coriobacteriaceae, Gemella, BVAB-1*, *Anaerococcus, Peptoniphilus* and *Finegoldia.*

A


Fuzzycluster
BV
A. vaginae
Megasphaera
Prevotella
Dialister
Sneathia
Aerococcus
Anaerococcus
Peptoniphilus
Finegoldia
P. timonensis
Clostridium
Parvimonas
Coriobacteriaceae
Gemella
Mycoplasma
BVAB1
L. jensenii
Lactobacillus
L. reuteri
Staphylococcus
Veillonella
P. anaerobius
S. anginosus
S. agalactiae
P. pallens
P. melaninogenica
L. delbrueckii
L. iners
G. vaginalis
L. crispatus
15
10
5
0
fuzzycluster
1
2
3
BV
Normal
Intermediate
BV

B

C


Bray Distance – PCoA by BV
Axis.2 [27.6%]
Axis.1 [48.9%]
0.6
0.4
0.2
0.0
−0.2
−0.4
−0.4
−0.2
0.0
0.2
0.4
0.6
fuzzycluster
1
2
3

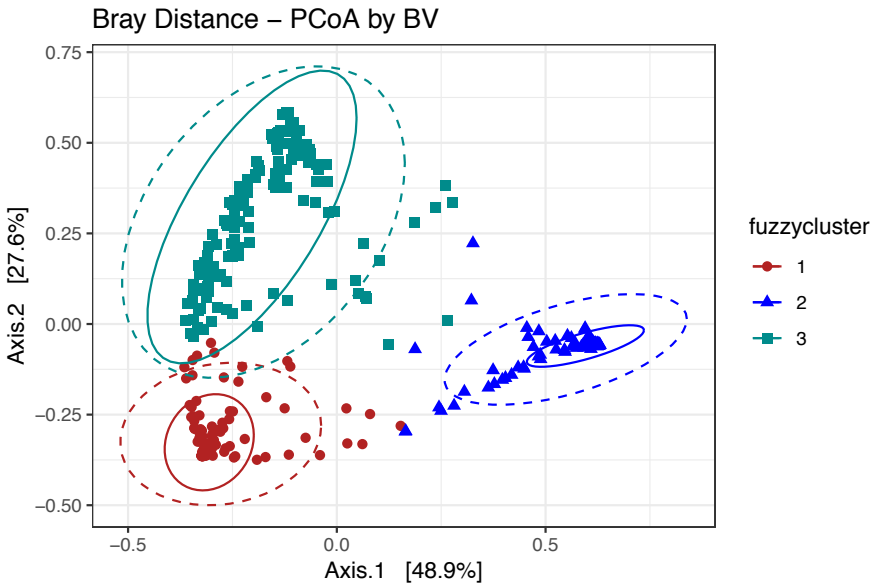


Figure 3. 5A Heatmap of unsupervised clustering using merged taxa according to Bray Curtis distance >0.01, showing the 30 most abundant taxa in all 356 pregnant women. Each column represents a woman and rows represent taxa. Red colour represents most abundant while blue presents least abundant or absence of $\log_2$ transformed OTU counts. **B & C** Principal coordinates (PCoA) plot, (Bray-Curtis Distances) showing three clusters coloured by fuzzy cluster. **B.** Fuzzy cluster 1 (CST1) - dominated by *Lactobacillus iners* shown in red, Fuzzy cluster 2 (CST2) - dominated by *Lactobacillus crispatus* shown in blue, Fuzzy cluster 3 (CST3) - dominated by *Gardnerella vaginalis* shown in green. **C.** Ellipses of a t-distribution (continuous) and a normal-distribution (dashed).

When comparing within sample microbial diversity (α-diversity) between the three CST groups, CST3 had significantly higher diversity by Shannon and Simpson measures, with Kruskal Wallis $p < 2.2e\text{-}16$ for both **(Figure 3.6A)**. Furthermore, comparison of beta diversity using non-metric dimensional analysis of Bray Curtis distances based on Adonis with 999 permutations revealed significant differences between CST1, CST2 and CST3 ($p < 0.001$; **Figure 3.6B).**

To visualize the most relatively abundant genera in the CST groups we used bar plots (including only taxa with genus or species level annotation). At genus level, higher relative abundances of *Gardnerella*, *Atopobium* and *Dialister* were observed in CST3 as compared to CST1 and CST2 as expected **(Figure 3.6C).** *Lactobacillus* was abundant in all CSTs, but there was notably higher relative abundance of *Gardnerella* in CST1 as compared to CST2. At species level the bar plot demonstrated the

dominance of *L. iners* in the 3 CSTs in this cohort as previously described **(Figure 3.6D)** and its ability to cohabit with BV associated microbiota (CST3)

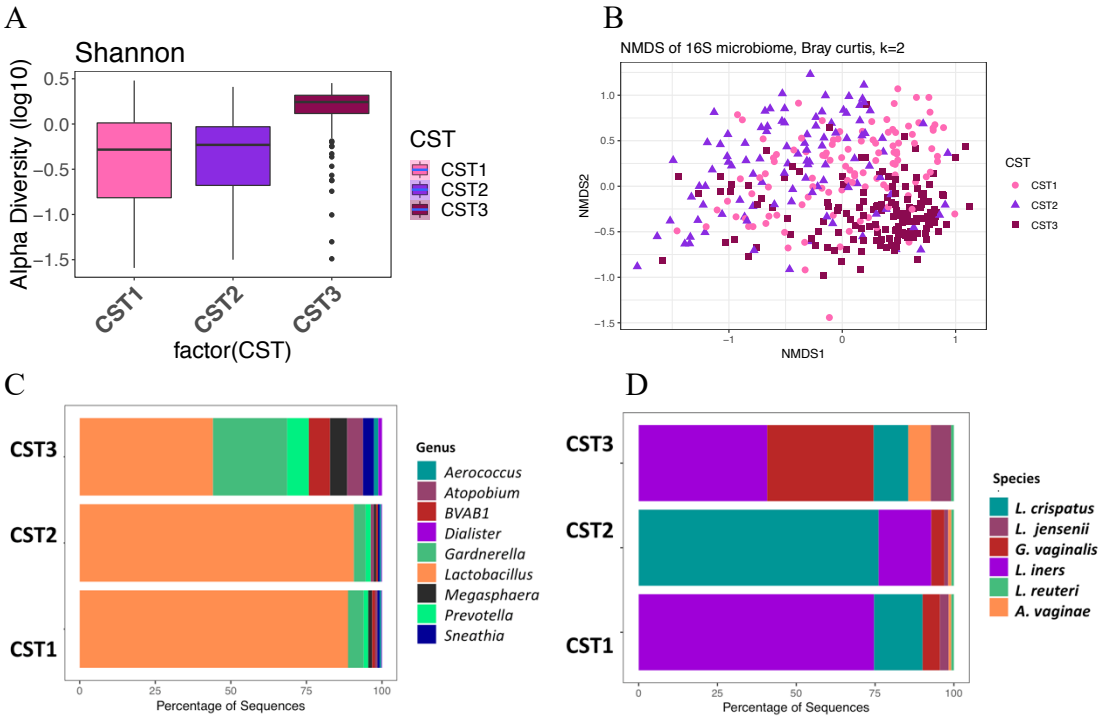


Figure 3. 6 Microbial diversity and clustering by CST status

A. Alpha diversity (Shannon, Kruskal Wallis p= < 2.2e-16)

B. Beta diversity (Bray Curtis Adonis p=0.001) comparing CST groups.

C. Bar plot showing relative abundance of microbial communities at genus level.

D. Bar plot showing relative abundance of microbial communities at species level using percentage of standardised sequence reads.

CST3 was the most prevalent, present in 145/356 (41%) of women, whereas 102/356 (29%) were CST2 and 109/356 (31%) were CST1 **(Table 3.2** and **Figure 3.5).**

Furthermore, women with CST3 had a mean vaginal pH of 4.40; higher than that of CST2 (4.01) and CST1 (4.13) (K. Wallis p or p<0.001 for both comparisons).However it is important to note that the magnitude of difference might not be clinically important. When comparing cohort characteristics according to CST in a univariate analysis, there were no significant differences in participant characteristics such as gestational age at swab collection, gravida, parity, maternal age, pregnancy induced hypertension, previous history of poor outcome, GBS carriage, partner smoker and antibiotic use between women in the three CSTs. However, statistically significant associations with CST were observed for HIV status (p =0.009), vaginal douching (p =0.016), vaginal pH (p <0.001) and BV (p <0.001) **(Table 3.2)**. CST3 was associated

with higher Nugent score and higher pH compared to other CSTs as expected. Moreover, HIV-infected women were less likely to be in CST2.

Table 3. 3 Cohort characteristics according to Community state types.

	CST1 n=109	**CST2 n=102**	**CST3 n=145**	**p-value**
Gestational age at collection, mean (SD)	27.5 (6.09)	28.6 (5.10)	28.9 (7.09)	0.186
Vaginal pH, mean (SD)	4.10 (0.57)	4.0 (0.59)	4.4 (0.77)	**<0.001**
Gravida, mean (SD)	2.9 (1.62)	2.9 (1.69)	2.8 (1.66)	0.775
Para, mean (SD)	1.3 (1.20)	1.5 (1.30)	1.2 (1.14)	0.266
Maternal age, mean (SD)	29.3 (6.45)	29.7 (6.13)	28.9 (6.60)	0.621
Pregnancy induced hypertension n (%)	5 (4.6)	8 (7.8)	8 (5.5)	0.586
Previous poor outcome n (%)	39 (35.8)	27 (26.5)	43 (29.7)	0.324
GBS n (%)	45 (41.3)	42 (41.2)	52 (35.9)	0.594
Partner smoker n (%)	8 (7.3)	8 (7.8)	10 (6.9)	0.961
Antibiotic use n (%)	18 (16.5)	12 (11.8)	15 (10.3)	0.326
HIV (%)	14 (12.8)	4 (3.9)	24 (16.6)	**0.009**
Trimester 2nd n (%)	45 (40.9)	32 (31.4)	51 (35.4)	0.346
Trimester 3rd n (%)	65 (59.6)	69 (67.6)	94 (64.8)	0.464
Nugent score, mean (SD)	1.7 (2.74)	1.3 (2.65)	5.5 (3.15)	**<0.001**
Vaginal douching, n (%)	41 (37.6)	53 (52.0)	80 (55.2)	**0.016**
Bacterial vaginosis (BV), n (%)				**<0.001**
BV positive (Nugent 7-10)	10 (9.2)	9 (8.8)	69 (47.6)	
Intermediate (Nugent 4-6)	13 (11.9)	7 (6.9)	37 (25.5)	
BV negative (Nugent 0-3)	86 (78.9)	86 (84.3)	39 (26.9)	

CST=community state type, GBS= group B Streptococcus, LBW =low birth weight,
SGA= small for gestational age

A multinomial regression model was performed to identify factors associated with CST including factors both in the literature and found associated in bivariate analysis such as HIV, gravida, previous poor outcomes and vaginal douching. CST2 (*L. crispatus* dominant) was used as the reference category and the results output was obtained using stargazer package in R (Hlavac, Marek; 2018). Vaginal douching was associated with CST1 ($p < 0.05$). The significant association between HIV Infection and CST1 and CST3 persisted in the multinomial model **(Table 3.4)**

Table 3. 4 Multinomial logistic regression of factors associated with bacterial vaginosis

	Dependent variable:	
	CST1	**CST3**
HIV	1.213** (0.602)	1.529*** (0.566)
Maternal age	-0.022 (0.026)	-0.029 (0.025)
Gravida	0.049 (0.099)	0.021 (0.095)
Vaginal douching	-0.482** (0.288)	0.253 (0.271)

Note: *p<0.1; **p<0.05; ***p<0.01 Odds ratio =number in brackets Beta-coefficient= number on top

3.3 Vaginal microbiota and bacterial vaginosis associated bacteria in pregnancy

There was a 75% agreement in BV classification by both Nugent and Hay Ison, therefore Nugent was chosen for downstream analysis as it is the most commonly used method in the literature. Interestingly, an equal proportion of women with normal Nugent scores fell within CST1 (86/211 (40.8%) and CST2 86/211 (40.8%). Though microbiological differences were evident, there were no differences in most demographic and clinical characteristics by BV status, including previous poor birth outcome (p=0.586), vaginal douching (p=0.591), pregnancy induced hypertension (PIH) (p=0.764), partner smoking status (p=0.228), GBS carriage (p=0.524), gestational age at collection (p=0.123) and parity (p=0.057) (**Table 3.5**). BV negative women tended to be older (30.3 years vs 27.9 for BV+ and 27.6 for intermediate BV), HIV-infected and have had more prior pregnancies.

Table 3. 5 Cohort characteristics according to BV status

	BV n=88	**Intermediate** n=57	**Normal** n=211	**p-value**
Gestational age at collection, mean (SD)	29.48 (7.87)	28.49 (5.62)	27.85 (5.64)	0.123
Partner smoker n (%)	7 (8)	7 (12.3)	12 (5.7)	0.228
HIV n (%)	18 (20.5)	7 (12.3)	17 (8.1)	**0.010**
Regimen n (%)				0.636
AZT 3TC	2 (11.8)	0 (0.0)	2 (12.5)	
TDF, FTC, EFV	15 (88.2)	7 (100.0)	13 (81.2)	
Blood CD4+ [cell/mm^3(n=24)] [median (IQR)]	524 (544-546)	495 (497-493)	509 (508-509)	0.590
CST n (%)				**<0.001**
CST1	10 (11.4)	13 (22.8)	86 (40.8)	
CST2	9 (10.2)	7 (12.3)	86 (40.8)	
CST3	69 (78.4)	37 (64.9)	39 (18.5)	
Vaginal douching n (%)	47 (53.4)	26 (45.6)	101(47.9)	0.591
Vaginal pH, mean (SD)	4.43 (0.83)	4.21 (0.57)	4.11 (0.62)	**0.001**
Gravida, mean (SD)	2.51 (1.49)	2.81 (1.53)	3.04 (1.73)	**0.038**
Para, mean (SD)	1.07 (1.08)	1.25 (1.21)	1.43 (1.24)	0.057
Maternal age, mean (SD)	27.87 (6.3)	27.55 (6.16)	30.25 (6.35)	**0.002**
Pregnancy induced hypertension n (%)	4(4.5)	3(5.3)	14 (6.6)	0.764
Previous poor outcome n (%)	24 (27.3)	16 (28.1)	69 (32.7)	0.586
History of previous preterm birth	11 (12.5)	7 (12.3)	35 (16.6)	0.554
GBS n (%)	33 (37.50	19 (33.3)	87 (41.2)	0.524
Inflammation (%)				**<0.001**
High	43 (50.0)	14 (25.0)	24 (13.2)	
Low	6 (7.0)	6 (10.7)	67 (36.8)	
Medium	37 (43.0)	36 (64.3)	91 (50.0)	

CST=community state type, GBS= group B Streptococcus. Women were grouped into three categories based on the concentrations of inflammatory cytokines and chemokines (IL-1β, IL-6, TNFα, IL-8, Eotaxin, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES) in their genital tracts by factor analysis as described in Masson *et al.* (2015). Women were grouped as having high inflammation if their inflammatory factor score was in the upper quartile (≥ 75th percentile), medium inflammation if their score was in the interquartile range (<75th - >25th percentile) and low inflammation if their score was in the lower quartile (≤25th percentile).

Maternal age (p=0.002), gravida (p=0.038), CST (p<0.001), vaginal pH (p=0.001) and HIV status (p=0.010) differed significantly by BV status **(Table 3.4)**. Women with normal Nugent scores were older, less likely to be HIV-infected, and had more prior pregnancies **(Table 3.5)**. A multivariate regression was performed to adjust for possible published confounding factors and factors that were significantly different in a univariate analysis (HIV, maternal age, vaginal douching and gravida). We excluded factors that were collinearly related to BV, such as CST and vaginal pH. After

multivariate logistic regression using BV as the outcome variable, HIV (p=0.003) was independently associated with BV (**Table 3.6**). This shows that BV positive women were 2.89 times more likely to be HIV-infected than BV negative women .

Table 3. 6 Multivariate logistic regression of factors associated with bacterial vaginosis

	Adjusted Odds Ratio	95 % CI	P-value
HIV	2.89	1.41-5.92	**0.003**
Maternal age	1.036	0.983-1.095	0.189
Vaginal douching	1.22	(0.74-2.00)	0.434
Gravida	1.171	0.947-1.464	0.152

CI= confidence interval, OR= odds ratio

As expected, comparing within sample microbial diversity (α-diversity) between the three BV groups (BV+, BV- and intermediate BV+), there was greater alpha diversity using both Shannon ($p < 2.2e-16$) and Simpson (Kruskal Wallis $p < 2.2e-16$) diversity measures in the BV+ group **(Figure 3.7A)**. Furthermore, comparison of beta diversity using non-metric dimensional analysis of Bray Curtis distances based on Adonis with 999 permutations revealed significant differences between BV, intermediate and negative samples **(**$p < 0.001$; **Figure 3.7B).** Unsupervised clustering of samples using Bray Curtis PCoA revealed clustering of BV positive samples with CST3, and BV negative samples in CST 1 and 2. The majority of women who had BV+ and intermediate microbiota by Nugent score clustered within CST3 (106/145 (73%), while 23 (15%) of the BV+ or intermediate clustered with CST1 and 16 (10%) with CST2 **(Figure 3.7; Table 3.3).**

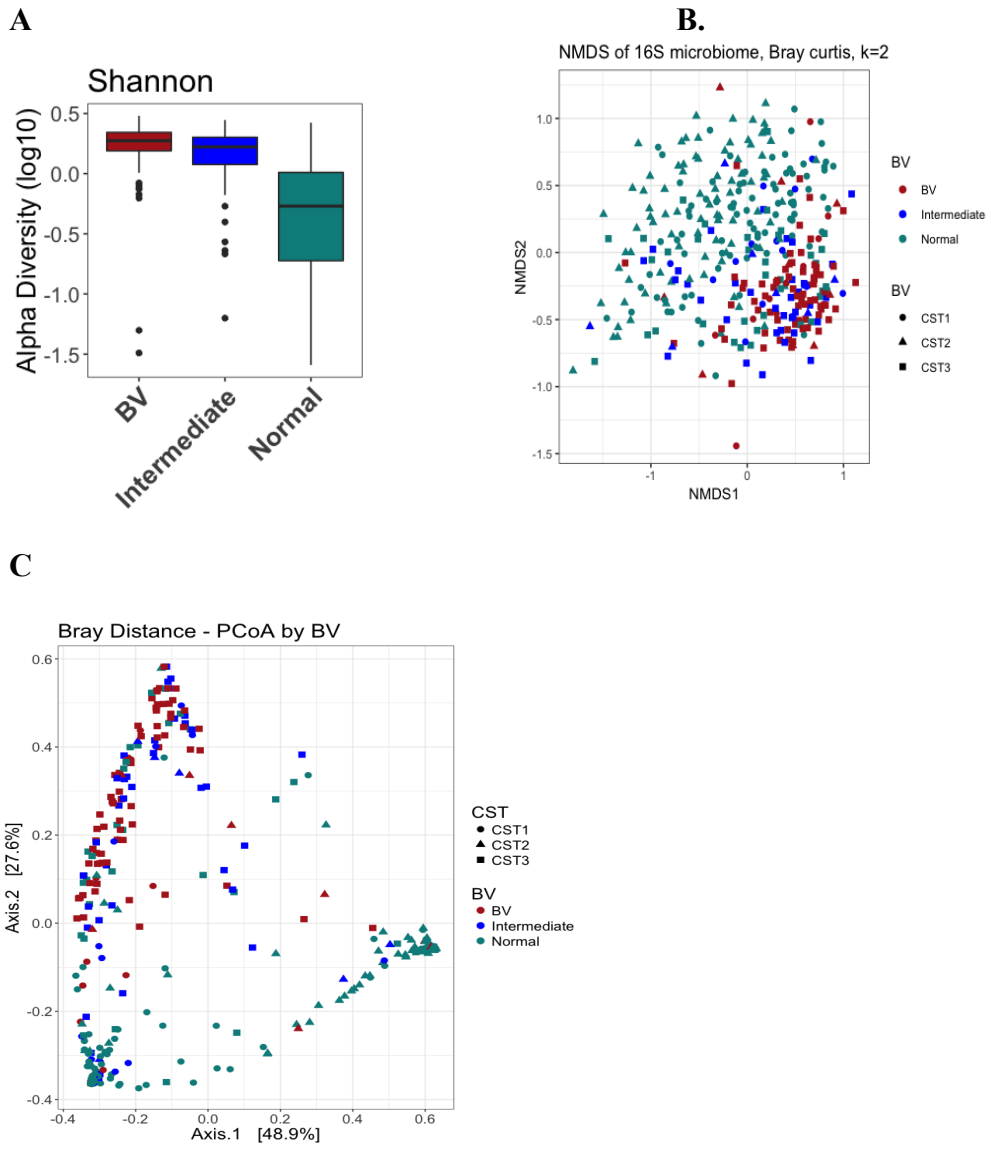


Figure 3. 7 Microbial diversity and clustering by BV status

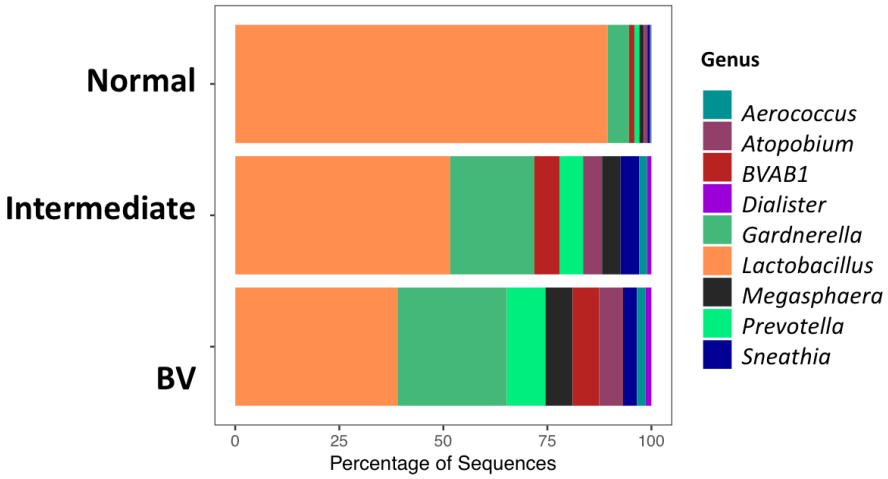
A. Alpha diversity (Shannon, Kruskal Wallis p= < 2.2e-16)

B. Beta diversity (Bray Curtis Adonis p=0.001) comparing BV+, intermediate and BV- women.

C. PCoA Clustering of BV classes according to CST types. BV and Intermediate cluster with CST 3 (*Gardnerella* dominated) p=<0.001.

To visualize the most relatively abundant genera in the BV groups, we used bar plots (**Figure 3.8A & B**). Notably higher relative abundances of *Gardnerella*, *Atopobium* and *Prevotella* in both the BV+ and intermediate BV+ groups were observed. *Dialister* and *Aerococcus* were drastically reduced in BV- women while *Lactobacillus* was the dominant genus in all three groups with a higher relative abundance in BV- women **(Figure 3.8A).** When looking at species relative abundance (including only those taxa with species-level annotation), As expected *L. crispatus* is more abundant in BV- (Normal) women as compared to intermediate and BV+ women while there is high abundance of *G. vaginalis* and *A. vaginae* in intermediate and BV+ women. **(Figure 3.7B).**

A



B

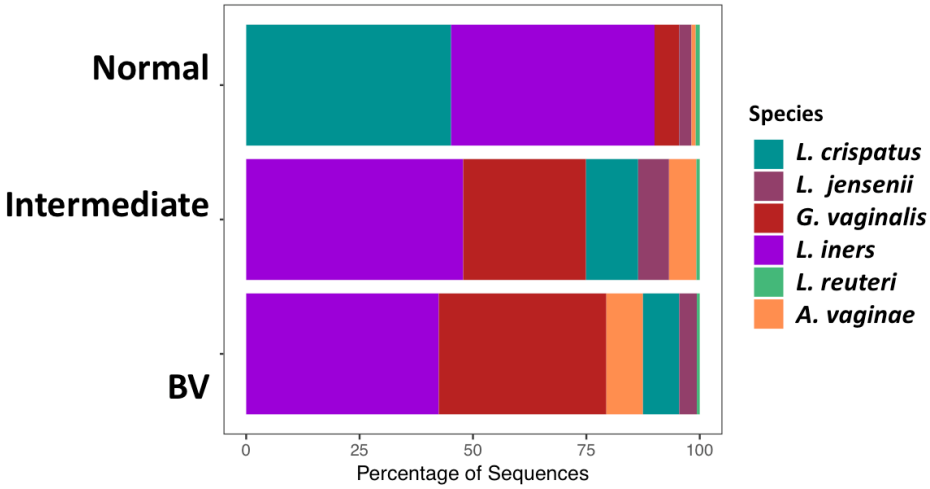


Figure 3. 9 A &B Bar plot showing relative abundance of microbial communities by BV status at **A** genus level and **B** species level using percentage of standardised sequence reads**.**

To explore which taxa were significantly differentially abundant between BV positive and negative status, metagenomeSeq analysis was performed on taxa merged at lowest taxonomic levels. Intermediate BV was not considered in order to get clear distinction between the BV+ and BV- groups, and because the model only allows for two group comparisons. Forty-five taxa met the threshold criteria of FDR adjusted p< 0.05, absolute fold change (FC) of 1.25 and percentage presence in at least one group of 20% **(Figure 3.9).** *Collinsella aerofaciens, Oscilllospira, Dialister, Blautia, Corynebacterium stationis, Ruminicoccus bromii, L. crispatus and coleohominis, Nevskia ramosa, G. vaginalis, A. vaginae,* to mention a few, were among taxa most differentially abundant between BV+ pregnant women and BV- pregnant women with adjusted p <0.0001. *L. iners* was not differentially abundant between the two groups.

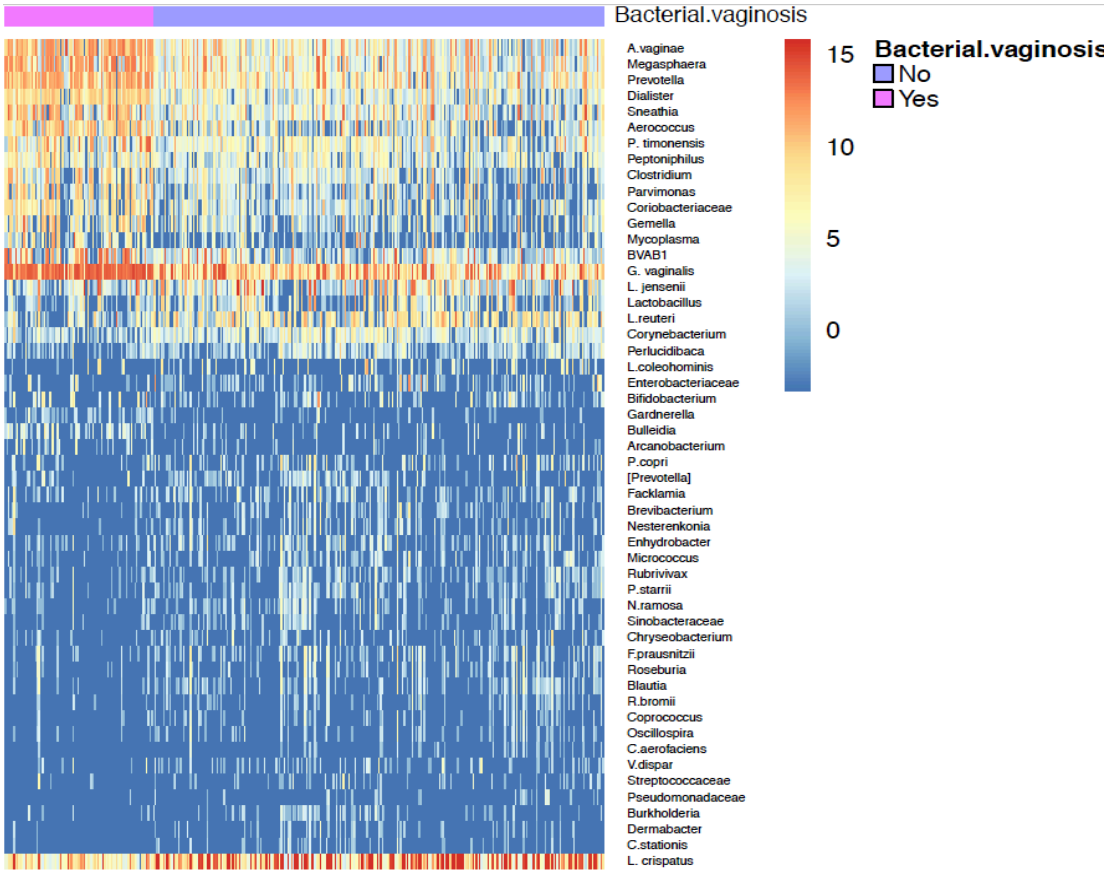


Figure 3.10 MetagenomeSeq supervised heatmap showing microbes differentially abundant between BV versus Normal vaginal microbiota. Differential abundance testing was performed on taxa merged at the lowest taxonomic level. Each column represents a woman and rows represent taxa. Red colour represents the most abundant while blue presents least abundant or absence of $\log_{10}$ transformed OTU counts.

Community profiling and metagenomeSeq results were reinforced by Random forest (RF) analysis **(Figure 3.10)** in distinguishing BV+ and BV- women. As with the other analyses, this analysis identified a high level of distinction of taxa distinguishing BV+ from BV-. The model resulted in a 10-fold cross validation error of 17.5% with the training set having a sensitivity of 68%, specificity 90% while the test set had a sensitivity of 69% and a specificity of 90%. Both sets had a similar degree of accuracy of 83% showing *Dialister* as the most predictive of BV in this cohort with a mean decrease Gini index of 7.4. The ten genera found to be most predictive of BV by random forest in order were *Dialister, Gardnerella, A. vaginae, Aerococcus, Megasphaera, Prevotella, Coriobacteriacae, Sneathia, Lactobacillus,* and *BVAB1.* **(Figure 3.10).** In this model, *L. iners* was among the taxa that distinguished BV+ from BV-.

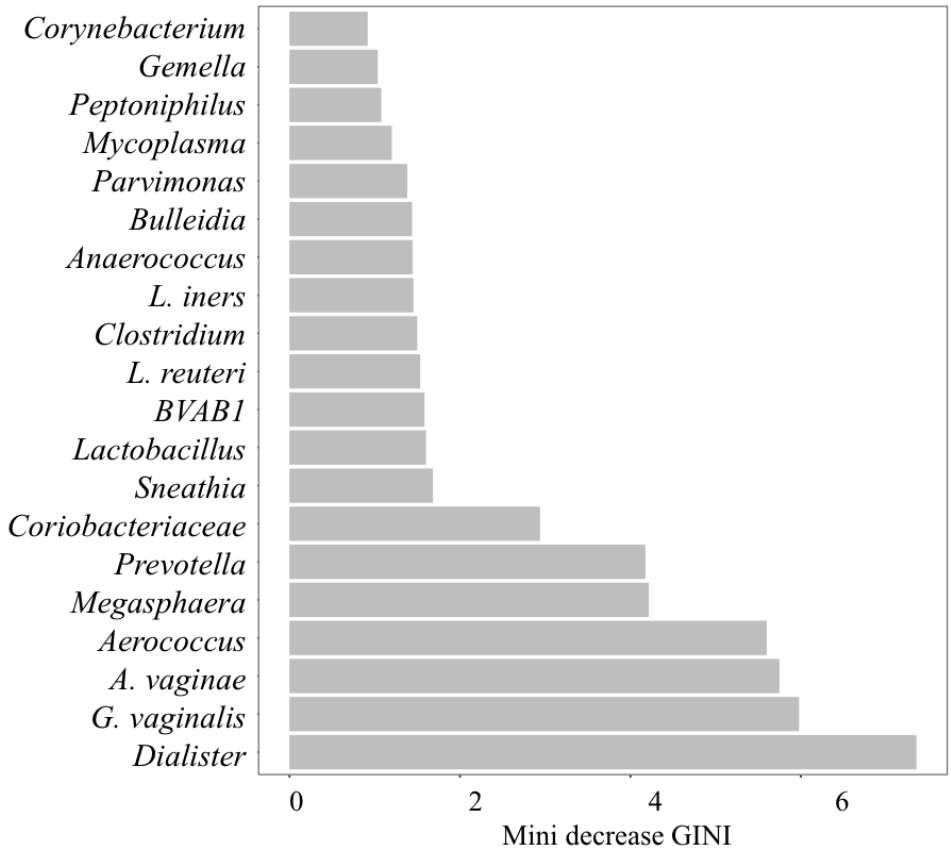


Figure 3.11 Random forest using taxa merged at the lowest taxonomic level showing the taxa predictive of bacterial vaginosis. The X-axis shows the mean decrease in the Gini index (length of bar represents relative importance of each taxon in the model).

3.4 Discussion

This chapter describes the vaginal microbiota of a cohort of Zimbabwean pregnant women, and its relationship with BV. Vaginal microbiota in both pregnant and non-pregnant women has been studied in different ethnic groups but is underexplored in pregnant African women.

In our study, examining the vaginal microbiota of pregnant women in this Zimbabwean cohort, three distinct clusters were evident; CST1 (*L. iners-dominant*), CST2 (*L. crispatus-dominant*), CST3 (*G. vaginalis-dominant*). Using exactly the same method as our study for both clustering (i.e. Fuzzy clustering) and sequencing of the V4 hypervariable region of the 16S rRNA gene, three main vaginal microbiome clusters were obtained in a South African study of non-pregnant adolescents by our group [134]. The main clusters in Lennard *et al.,* (2018) were *L. iners*- dominated, *L. crispatus-dominated* and one diverse microbiota cluster. In research conducted in non-African women, various clusters of vaginal microbial communities have been described, although methods of clustering and target variable regions differ.

Two main vaginal microbial clusters were described in Chinese women when sequencing the V3 hypervariable region [149], as opposed to five main clusters in North American women using data from sequencing of the V1-V2 hypervariable regions [51]. In sub Saharan African studies, different vaginal microbial clusters have been described, however studies also varied in the hypervariable region used for sequencing and the method used to define a cluster. Eight clusters were described in Tanzanian women using sequencing of the V6 hypervariable region using similarity of organism abundance and the similarity was visualized using neighbor joining trees [145]. Anahtar and colleagues found four "cervicotypes" in young South African women, however the clustering method was not clear as they report to have used the dominant bacterial species [369]

At the phylum level, Firmicutes and Actinobacteria were the most abundant in the vaginal microbiota of these pregnant women, similar to that reported in previous studies in of non-pregnant [98, 118] and pregnant women [160]. Romero *et al.,* (2014) reported that the vaginal microbiota during pregnancy was relatively healthy and stable, and that it would most often shift from one community dominated by one *Lactobacillus spp* to another dominated by another *Lactobacillus spp* [58]. This agrees with Freitas *et al.,*

(2017*)* who reported that CSTs dominated by *Gardnerella* and *Atopobium* were not detected in pregnant as often as in non-pregnant women [6]. Our findings are divergent in that over half of the pregnant women in this cohort had a diverse vaginal microbial community (CST3; *Gardnerella-dominant*) with the remaining half almost equally shared between CST1 and CST2 (*Lactobacillus dominant*). It seems that a dysbiotic environment is characteristic of African pregnant women, however, genetic, cultural practices such as douching, and behavioural differences could be contributing factors. There is a need to consider a longitudinal study with multiple time points in order to further investigate the influence of these factors on the vaginal microbiota during pregnancy.

Of the *lactobacilli* species, we found *L. iners* to be the most dominant in the vaginas of Zimbabwean pregnant women and was the taxon present in more than 50% of the samples regardless of Nugent score. *L. iners* is consistently found present in vaginal microbiota of African communities and women of African descent, and seems to comfortably coexist where BV-associated organisms dominate, unlike other *Lactobacillus spp* [24, 57, 133, 257]. Lactobacillus spp. other than *L. iners* produce the D-lactic acid isoform which may inhibit anaerobic bacteria preventing development of dysbiosis, while *L. iners* produce L-lactic acid isoform which inhibits only a few organisms such as HIV [67-70]. Based on this notion, since our cohort had a high abundance of *L. iners,* the production on L-lactate isoform would not inhibit the proliferation of anaerobic organisms thus leading to high abundance of dysbiosis causing vaginal imbalance. Other studies have found *L. iners* to be ubiquitous in non-African women also, present both in normal healthy microbiota and in dysbiotic environments, confirming further need of study to ascertain the role played by this bacterium in vaginal health [51, 85, 130, 257].

Romero *et al.,* (2014) found *L. vaginalis*, *L. crispatus*, *L. gasseri* and *L. jensenii* abundant in pregnant North American women, regardless of ethnicity, while they found very low relative abundance of *L. iners*. This further demonstrates the geographical differences between the vaginal composition of African women compared to other groups. More so, in our study of normal asymptomatic pregnant women we observed that vaginal microbiota of more than 50% of the women were *G. vaginalis* dominated regardless of BV status. Consistent with our study, Machado *et al.,* (2017) found 67% of Portuguese pregnant women colonised with *G. vaginalis* despite the low prevalence

of clinically diagnosed BV [376]. Although the role of *L. iners* has not been established to our knowledge, a high level of coexistence of *L. iners* and *G. vaginalis* was observed in our cohort. In a review by Petrova *et al*., (2017) reports a symbiotic relationship which might exist between *L. iners* and *G. vaginalis* and has also observed that *L iners* gene encodes for a pore forming toxin (inerolysisn) related to vaginolysisn by *G. vaginalis* [67]. There is need for further studies to establish the relationship between these two taxa in the vagina and the role that *L. iners* might play in the vaginal environment.

The association of HIV with dysbiotic vaginal microbiota (CST3) was evident in this cohort. This is in agreement with previous studies done in Africa, albeit in non-pregnant African women [143, 167]. On the other hand, a diverse cervicovaginal microbiota was reported to be one of the risk factors for with HIV acquisition [9, 135]. Therefore, it remains unclear whether HIV changes the microbiota, or dysbiosis is enriched in HIV-infected women since it was what put them at risk for acquisition in the first place. However, in our study we are not able to explain which one posed risk to the other because we did not follow up HIV acquisition, since the women were already HIV positive at collection. Even though our study was a cross sectional study which cannot assess temporal relationships between HIV and BV, our observations reiterate previously published findings of an increased BV prevalence in women with HIV [154, 165, 170]

A diverse milieu of bacterial species is characteristic of BV, usually associated with poor health outcomes, although the mechanisms of BV are still poorly understood [85, 158]. The presence of *G. vaginalis* was believed to be characteristic of BV+ status before the advent of molecular methods to more deeply characterise the vaginal microbiota [122, 149, 241]. Numerous studies since agree that there is no one organism that defines BV, as previously was thought [117]. Different taxa in different sub-populations singly or in combination have been reported to be biomarkers of BV, such as *G. vaginalis* and *A vaginae* [120] in France pregnant women, *Megasphaera type I, BVAB2, A. vaginae and G. vaginalis [248]* in university students and *G. vaginalis*, *A. vaginae*, *Eggerthella*, *Prevotella*, BVAB2, *Megasphaera* [122] and BVAB1 and BVBA2 [267, 377] in non-pregnant reproductive aged women. In our study of pregnant women, *Dialister* was the main predictive genus of BV as confirmed by RF and it was also among the most significantly differentially abundant by metagenomeSeq, followed

by *Gardnerella*, *A. vaginae, Megasphaera, Prevotella* and *Aerococcus.* Testing for *Dialister* could be explored in further studies as a potential test for BV in pregnancy in Zimbabwean women especially in areas where microscopy is not available.

In summary, here we find a high prevalence of vaginal dysbiosis in pregnant Zimbabwean women, which highlights the importance of BV screening during antenatal visits.

Chapter 4: Vaginal microbiota and birth outcomes

Reproductive health outcomes are likely to be influenced by the composition and interactions of the maternal vaginal microbiome throughout pregnancy [150]. BV has been associated with several pregnancy complications such as preterm labour, PROM, LBW, preterm delivery [18, 20] and possibly spontaneous abortion [19, 148]. Additionally, BV may increase susceptibility to sexually transmitted infections such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis [64]*, *Trichomonas vaginalis [148]*, HSV-2 [151] and HIV [152-154] which are also associated with poor birth outcomes.

This chapter describes the vaginal microbiota of Zimbabwean pregnant women in relationship to birth outcomes. The vaginal microbiota is a key component of the defense system which confer protection against microbial and viral pathogens [216]. Nevertheless, vaginal microbiota undergoes significant changes during pregnancy due to metabolic and physiologic alterations that occur in a woman from conception to delivery [378, 379].

Numerous studies have been conducted on the composition of vaginal microbiome and its role in health outcomes [6-8, 380,Dunlop, 2015 #11]. Nonetheless, contrasting evidence has been reported regarding the vaginal microbiota and pregnancy outcomes, where some studies have found that vaginal microbiome is different [7, 381] or similar [58] between women who experience adverse versus normal birth outcomes. Several reports have demonstrated associations between vaginal microbiome and preterm birth, small for gestational age (SGA) and low birth weight (LBW) infants [6, 380]. Specific organisms, such as *Mycoplasma hominis*, *G. vaginalis* and/or *A. vaginae*,BVAB1, *Megasphaera* phylotype 1, and *Leptotrichia/Sneathia* spp. in the vaginas of pregnant women have been reported to be associated with adverse birth outcomes[157, 160] However, Foxman *et al.,* (2014) reported that *Mageebacillus indolicus* (BVAB3) was associated with a reduction in preterm delivery risk among BV+ women and thus concluded that some BV-associated organisms are not associated with poor birth outcomes [158]. Although there are some contradicting findings, some of the variability can be attributed to racial, ethnic and geographical differences leading to no conclusive evidence whether a relationship exists between the vaginal microbiome and pregnancy outcome. To our knowledge, few studies have been conducted in pregnant women of sub-Saharan Africa, therefore, our study seeks to describe findings on vaginal microbiota in relation births outcomes in this population.

4.1 Vaginal microbiota and preterm birth

Out of the 356 sequenced samples, 97 women (27%) were lost to follow up, There were 12 (3.4%) still birth deliveries, 1 (0.2%) miscarriage, 49 (13.8%) preterm births and 197 (55%) term deliveries. Of these, 244 women had singleton deliveries, complete data on birth outcomes and microbiota, 202 (83%) had term deliveries while 42 (17%) delivered preterm **(Table 4.1).** There were no differences in participant characteristics such as parity, HIV status, GBS status, BV status, vaginal pH, gestational age at collection or partner smoking status between preterm and term deliveries **(Table 4.1).** History of preterm delivery was not significantly predictive of PTB in the current pregnancy ($p=0.533$). Women who delivered preterm were more likely to be older ($p=0.007$), have LBW infants ($p<0.001$) and be multigravida ($p=0.006$) than women who experienced term deliveries.

Table 4.1 Characteristics stratified by preterm delivery

	Term n=202 (%)	**Preterm n=42 (%)**	**p-value**
Gestational age at collection in weeks, mean (SD)	28.8 (6.7)	28.7 (4.72)	0.941
pH, mean (SD)	4.2 (0.72)	4.28 (0.63)	0.541
Gravida, mean (SD)	2.9 (1.65)	3.6 (1.85)	**0.006**
Maternal age in years, mean (SD)	29.3 (6.37)	32.3 (6.64)	**0.007**
Parity n (%)	59 (29.2)	7 (16.7)	0.141
Partner smoker n (%)	13 (6.4)	1 (2.4)	0.507
HIV-infected n (%)	20 (9.9)	9 (21.4)	0.066
Regimen n (%)			NaN
AZT 3TC	0 (0.0)	3 (16.7)	
TDF, FTC, EFV	9 (100.0)	15 (83.3)	
Blood CD4+ [cell/mm^3(n=24)] [median (IQR)]	488.25 (481-495)	450.43 (445.25-454.75)	0.590
SGA n (%)	21 (11)	9 (21.4)	0.116
LBW n (%)	10 (5.2)	25 (59.5)	**<0.001**
Vaginal douching n (%)	98 (48.5)	20 (47.6)	1
CST n (%)			0.668
1	58 (28.7)	10 (23.8)	
2	59 (29.2)	15 (35.7)	
3	85 (42.1)	17 (40.5)	
Pregnancy induced hypertension n (%)	10 (5)	5 (11.9)	0.176
Previous poor outcome n (%)	58 (28.7)	18 (42.9)	0.106
History of previous preterm delivery	28 (13.9)	8 (19.0)	0.533
GBS n (%)	74 (36.6)	16 (38.1)	0.998
Nugent score, mean (SD)	2.4 (3.09)	3.3 (3.51)	0.145
BV n (%)			0.442
BV	52 (25.7)	7 (16.7)	
Intermediate	33 (16.3)	7 (16.7)	
Normal	117 (57.9)	28 (66.7)	

LBW= low birth weight, GBS= group B streptococcus, BV=bacterial vaginosis, CST= community state type, SGA= small for gestational age

A multivariate logistic regression was performed including characteristics significantly associated with preterm delivery in the univariate analysis. Additionally, published factors such as gravida, maternal age, antibiotic use [382], gestational age at collection, HIV, and vaginal microbiota [8] were also included. BV and CST were adjusted for separately due to their collinear relationship, however, no significant associations were observed between preterm delivery and either BV or CST. Table 4.2 shows the model

with BV diagnosed by Nugent score. Pregnancy induced hypertension (p=0.04) [Odds ratio 3.66 (1.10- 12.33)] and HIV (p=0.03) [Odds ratio 3.18 (1.11- 8.74)] remained significantly associated with preterm birth in the multivariate model **(Table 4.2)**.

Table 4.2 Multivariate logistic regression of factors associated with preterm delivery

	Adj. OR	**95% CI**	**p-value**
Gravida	1.08	0.79-1.47	0.62
Maternal age	1.04	0.97-1.11	0.23
Antibiotic use	0.50	0.15-1.40	0.22
Previous poor outcome	1.93	0.81-4.53	0.13
BV by Nugent score	1.08	0.97-1.21	0.20
Pregnancy induced hypertension	3.71	1.03-12.39	**0.04**
HIV	3.05	1.09-8.20	**0.03**
Parity	0.66	0.18-2.28	0.52

CI= confidence interval, OR=odds ratio

Microbial diversity comparisons showed no significant differences in alpha or beta diversity between women who experienced preterm deliveries as compared to term deliveries (Shannon; Kruskal Wallis p=0.37**,** Bray Curtis, Adonis=0.544) respectively **(Figure 4.1A & B).** Neither were there differences in CST distribution between women who delivered preterm versus term.

In this study, exact gestational ages at collection of samples may have been inaccurate, because most women were not able to access fetal ultrasound scans. Therefore, gestational age estimation was based on last menstrual period dating and/or fundal height. This could have introduced inaccurate determination of preterm deliveries in the 35- 37 weeks of gestation zone. Therefore, due to this limitation we also performed an analysis using three categories; early preterm birth (EPTB; <35weeks of gestational age, n=16), late preterm birth (LPTB; 35-36.6 weeks, n=26) and term birth (≥ 37wks, n =189). There were no differences in gestational age at collection (p = 0.191), HIV status (p = 0.081), partner smoker status (p = 0.417), nor pregnancy induced hypertension (p=0.148) between these three groups. BV was not associated with increased risk of EPTB (OR 1.18; 95%CI 0.55- 2.46) nor late preterm birth (LPTB) (OR 0.60; 95%CI 0.30- 1.33) in this cohort. When comparing the means of the vaginal pH of the three groups: LPTB (4.42), EPTB (4.06), and term birth (4.19), no significant

difference in mean vaginal pH was evident (P=0.20, Kruskal Wallis). These findings were coherent with the findings of binarised analysis (term versus preterm).

Consistent with the binarised comparisons, within sample diversity (alpha diversity) and beta-diversity of the vaginal microbiota between women who had EPTB, LPTB and term showed no significant differences (Shannon diversity (Kruskal Wallis p= 0.47), Bray Curtis distances (Adonis P=0.939) **(Figure 4.1C & D)**. Similarly, there were no differences in CST distribution between the groups.

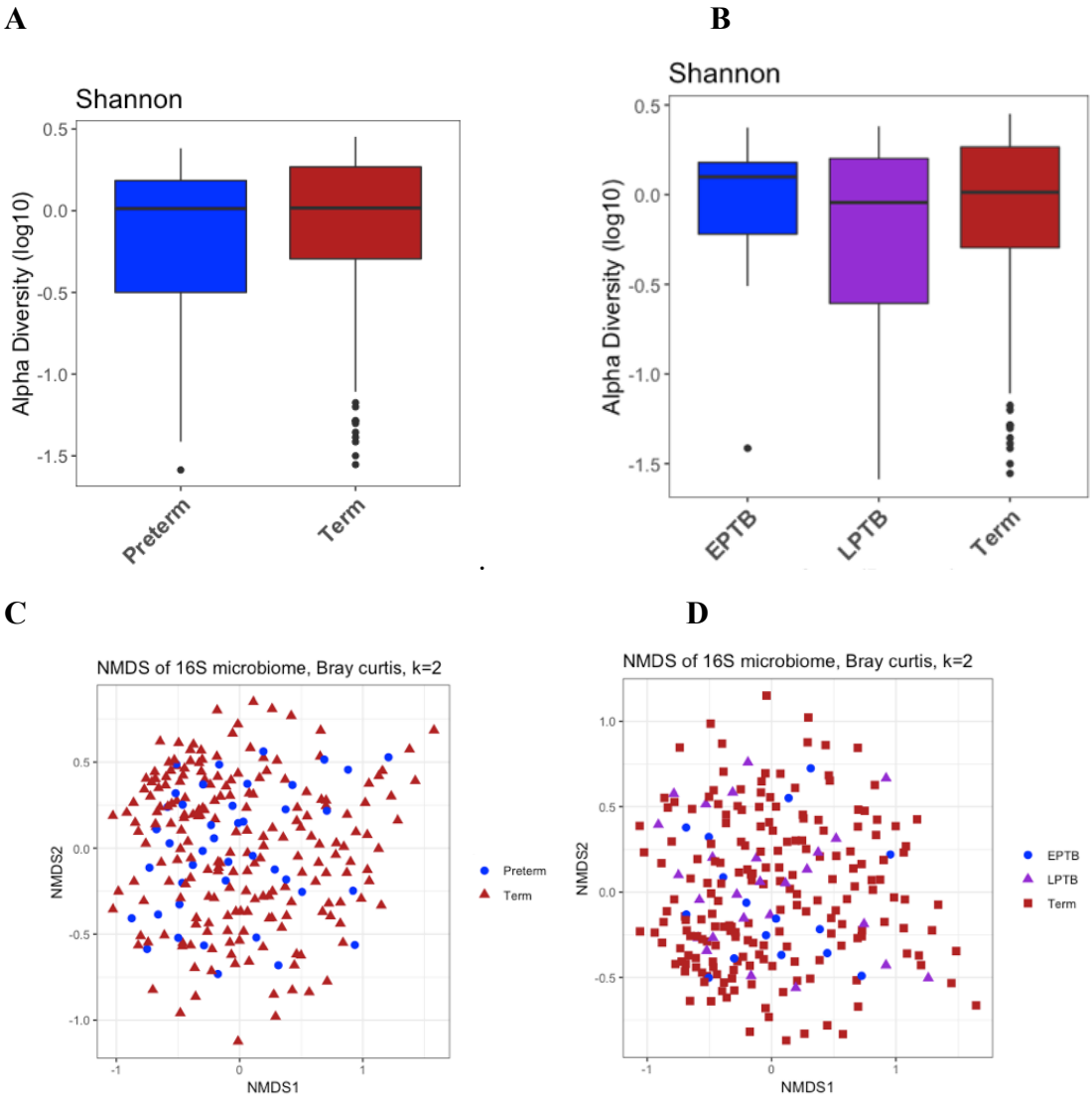


Figure 4.1 Diversity plots by preterm

- **A.** Shannon Alpha diversity between the preterm and term deliveries (p = 0.37),
- **B.** Shannon Alpha diversity between EPTB, LPTB and term deliveries (p = 0.47)
- **C.** Bray Curtis beta diversity between preterm and term deliveries (Adonis=0.544)
- **D.** Bray Curtis beta diversity between EPTB, LPTB and term deliveries (Adonis=0.94)

(Preterm <37 weeks, EPTB <35weeks, LPTB; 35wks - 36.6 weeks, term birth ≥ 37wks)

On observation of bar plots at genus level it was apparent that relative abundance of *Lactobacillus* was dominant and an almost equal proportion of *Aerococcus Atopobium, Megasphaera, Prevotella, BVAB-1*, *Sneathia* and *Gardnerella* were present in preterm and term births **(Figure 4.2A & B).** Relative abundance of *Gardnerella* and *A. vaginae* were high in women who experienced term deliveries while *L. iners* was more abundant (and conversely *L. crispatus* less abundant) in women who experienced preterm deliveries in the binary analysis, and EPTB in the three-group analysis **(Figure 4.2C & D).** These taxa had higher mean standardised read counts in healthy normal pregnancies as compared preterm birth pregnancies although these differences were not statistically significant (Wilcoxon rank sum test with continuity correction p >0.5) and welch t-sample (p > 0.45).

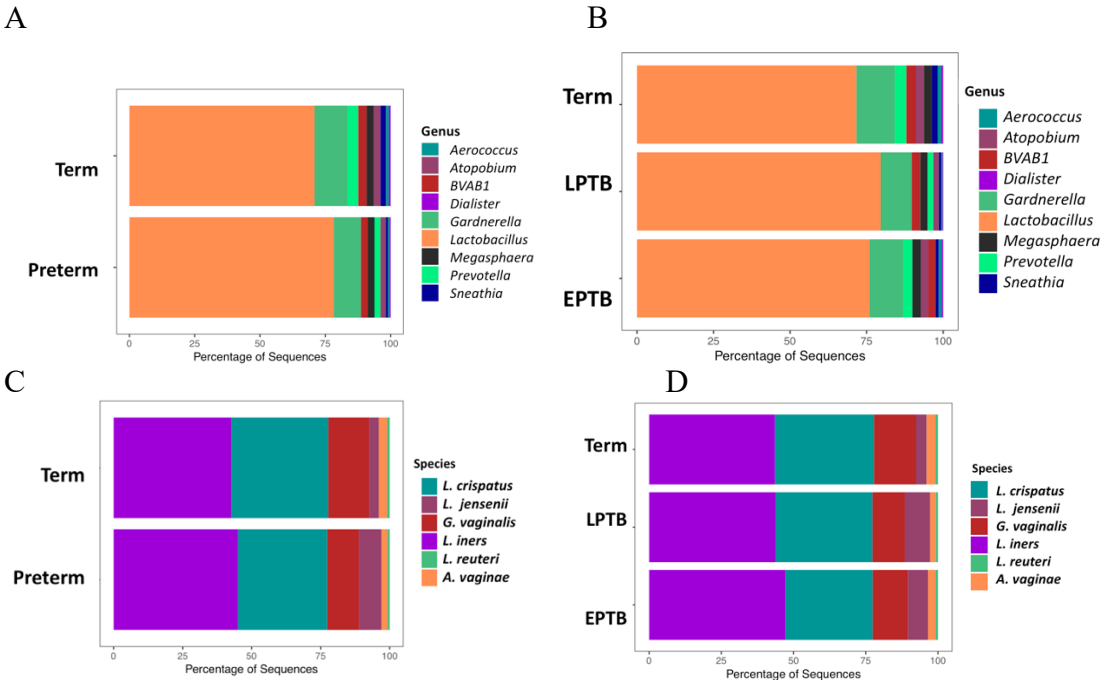


Figure 4.2 Bar plots by preterm categories.

A & B Relative abundance using percentage sequences at genus level

C & D Relative abundance using percentage sequences at species level

We then did a differential abundance testing using metagenomeSeq on the binarized groups (preterm versus term) and found that five taxa were significantly different in relative abundance between preterm and term birth. These taxa met the threshold criteria of FDR adjusted p= 0.05, absolute FC 1.25 and percentage presence in at least one group of 20%. Four taxa were significantly more abundant in preterm birth as compared to term were *[Mogibacteriaceae]* (adj. p=3.05e-07), *Lactobacillus coleohominis* (adj. p=1.17e-05), *V. dispar* (adj. p=5.55e-05), *Fusobacterium* (adj. p=0.0002) while *P. pallens* (adj. p=0.002) was more abundant in term than preterm **(Figure 4.3).** When attempting to use random forest model to predict taxa influential in preterm delivery, this model had poor specificity and positive predictive values were below 5%.

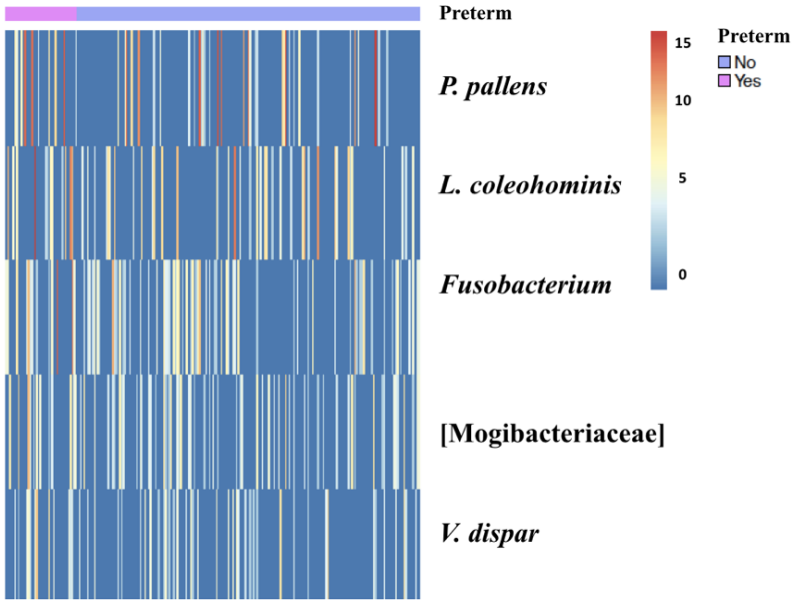


Figure 4.3 Differentially abundant vaginal taxa merged at the lowest taxonomic annotation by preterm using metagenomeSeq. Each column represents a woman and rows represent taxa. Red colour represents the most abundant while blue presents least abundant or absence of log_2 transformed OTU counts.

4.2 Vaginal microbiota and low birth weight

Low birth weight is defined as infants delivered with a birthweight below 2.5kg (<2500g). Infants who experience intrauterine growth retardation (IUGR) due to pathological processes do not reach their growth potential *in-utero* and are then delivered as LBW and/ or SGA even if they are born after 37 weeks (term) [383]. On the other hand, most preterm infants are usually delivered before they reach the weight of a term infant, and therefore are born LBW, although they may be appropriate for gestational age (AGA). Therefore, it is necessary to analyse LBW, SGA and preterm birth separately [383, 384], although LBW incorporates many of the adverse birth outcomes together.

This analysis is based on 233 women with complete sequencing, birth weight, and birth outcomes data. Nine women who had still birth and 2 women who had term deliveries did not provide birth weight data were excluded. Of the 233 women, 35 (15%) delivered low birth weight (LBW) babies, while 198 (85%) delivered term infants 2.5kg and above (Normal birth weight; NBW). Comparable participant characteristics between the two groups were gestational age at collection, parity, partner smoking status, HIV status, vaginal douching, vaginal pH, gravida, maternal age and GBS carriage ($p > 0.07$). However, there were significant differences in BV prevalence ($p=0.026$), PIH ($p=0.001$), previous history of poor outcome ($p=0.034$), SGA ($p<0.001$), and preterm delivery in the current pregnancy ($p < 0.001$) between women who delivered LBW versus NBW infants **(Table 4.3).**

Women who delivered NBW infants were significantly more likely to have BV than those who delivered low birth weight infants (53/198 (26.8%) compared to 2/35 (5.7%). Women who delivered LBW infants were more likely to have delivered preterm (26/198 (74.3%) versus 17/35 (8.6%), have had previous poor pregnancy outcomes (17 (48.6%) versus 57 (28.8%), have delivered SGA infants (15 (42.9%) versus 15 (7.6%) and had pregnancy induced hypertension during this pregnancy (7 (20%) versus 7 (3.5%). Women who experienced a previous preterm birth were more likely to deliver a low birth weight infant; 10 (28.6%) compared to women who delivered a NBW; 25 (12.6%).

Table 4.3 Cohort characteristics by low birth weight delivery

	NBW n =198	**LBW** n =35	**p- value**
pH, mean (SD)	4.2 (0.7)	4.3 (0.62)	0.415
Gravida, mean (SD)	2.9 (1.66)	3.5 (1.99)	0.071
Para, mean (SD)	1.3 (1.25)	1.5 (1.24)	0.365
Maternal age in years, mean (SD)	29.2 (6.66)	31.1 (4.84)	0.12
SGA n (%)	15 (7.6)	15 (42.9)	**<0.001**
Preterm n (%)	17 (8.6)	26 (74.3)	**<0.001**
BV n (%)			**0.026**
BV	53 (26.8)	2 (5.7)	
Intermediate	31 (15.7)	7 (20)	
Normal	114 (57.6)	26 (74.3)	
Pregnancy induced hypertension n (%)	7 (3.5)	7 (20)	**0.001**
Previous poor outcome n (%)	57 (28.8)	17 (48.6)	**0.034**
History of previous preterm delivery	25 (12.6)	10 (28.6)	**0.029**
Gestational age at collection in weeks, mean (SD)	28.89 (6.66)	28.69 (4.61)	0.863
Partner smoker status n (%)	11 (5.6)	1 (2.9)	0.802
HIV n (%)	21 (10.6)	7 (20)	0.196
Regimen n (%)			NaN
AZT 3TC	0 (0.0)	3 (16.7)	
TDF, FTC, EFV	9 (100.0)	15 (83.3)	
Blood CD4+ [cell/mm^3(n=24)] [median (IQR)]	488 (481-495)	450 (445-454.)	0.590
CST n (%)			0.094
1	52 (26.3)	14 (40)	
2	58 (29.3)	12 (34.3)	
3	88 (44.4)	9 (25.7)	
GBS n (%)	70 (35.4)	13 (37.1)	0.99
Vaginal Douching n (%)	99 (50)	12 (34.3)	0.125

LBW= low birth weight, GBS= group B streptococcus. BV=bacterial vaginosis, CST= community state type.
SGA= small for gestational age

A multivariate logistic regression analysis was performed including characteristics that were associated with LBW in the univariate analysis, as well as some factors associated with LBW in the literature such as gravida, parity [385, 386], maternal age [387], smoking partner [388], HIV infection and antibiotic use [382]. After multivariate logistic regression, BV (OR 0.82; 95% CI 0.70-0.94), prior history of poor birth outcome [Odds ratio (OR) 3.27; 95% CI 1.21, 9.00), HIV [OR 4.28; 95% CI 1.28, 13.64)] and pregnancy induced hypertension (PIH) [OR 12.63; 95% CI 3.29, 51.77)]

were all independently associated with LBW **(Table 4.4).** Among these factors PIH had the greatest effect on delivery of a LBW infant (p<0.001).

Table 4.4 Multivariate logistic regression of factors associated with low birth weight delivery

	Adj. OR	95% CI	P value
Gravida	1.08	0.78-1.55	0.45
Maternal age	1.00	0.92-1.08	0.94
Antibiotic use	0.82	0.24-2.47	0.66
Nugent score	0.82	0.70-0.94	0.009
Previous poor outcome	3.01	1.15-8.03	**0.02**
Pregnancy induced hypertension	12.63	3.29-51.77	**<0.001**
HIV	4.28	1.28-13.64	**0.02**
Parity	0.76	0.18-3.00	0.96
Smoking partner	0.33	0.01-2.54	0.42

CI= confidence interval, OR=odds ratio, BV= Bacterial vaginosis

Alpha diversity of the vaginal microbiota was significantly higher in women who delivered NBW versus LBW infants when calculated using both Shannon and Simpson diversity (p=0.02 and 0.001 respectively) **(Figure 4.5 A).** Between sample (Beta) diversity showed a trend towards a significant difference between samples with LBW and those without using Bray Curtis measures (p=0.055) **(Figure 4.5 B)**. There was a trend towards more women with CST 1 (*L. iners*-dominated) vaginal microbiota delivering LBW and women with CST-3 (*G. vaginalis*-dominated) delivering NBW infants (p=0.094).

Using bar plots to visualize mean relative abundance, a higher relative abundance of the *Lactobacillus* genus was observed in women who experienced LBW deliveries as compared to women who delivered NBW **(Figure 4.5 C)**. At species level, *L. iners* and *L. jensenii* were increased in abundance in the LBW group with a corresponding decreased relative abundance of *A. vaginae* and *G. vaginalis* compared to NBW group. Interestingly, *L. crispatus* appeared at the same relative abundance regardless of infant weight. This might imply a specific influential relationship between *L. iners* and LBW delivery as compared to other *Lactobacillus* species as depicted at genus level.

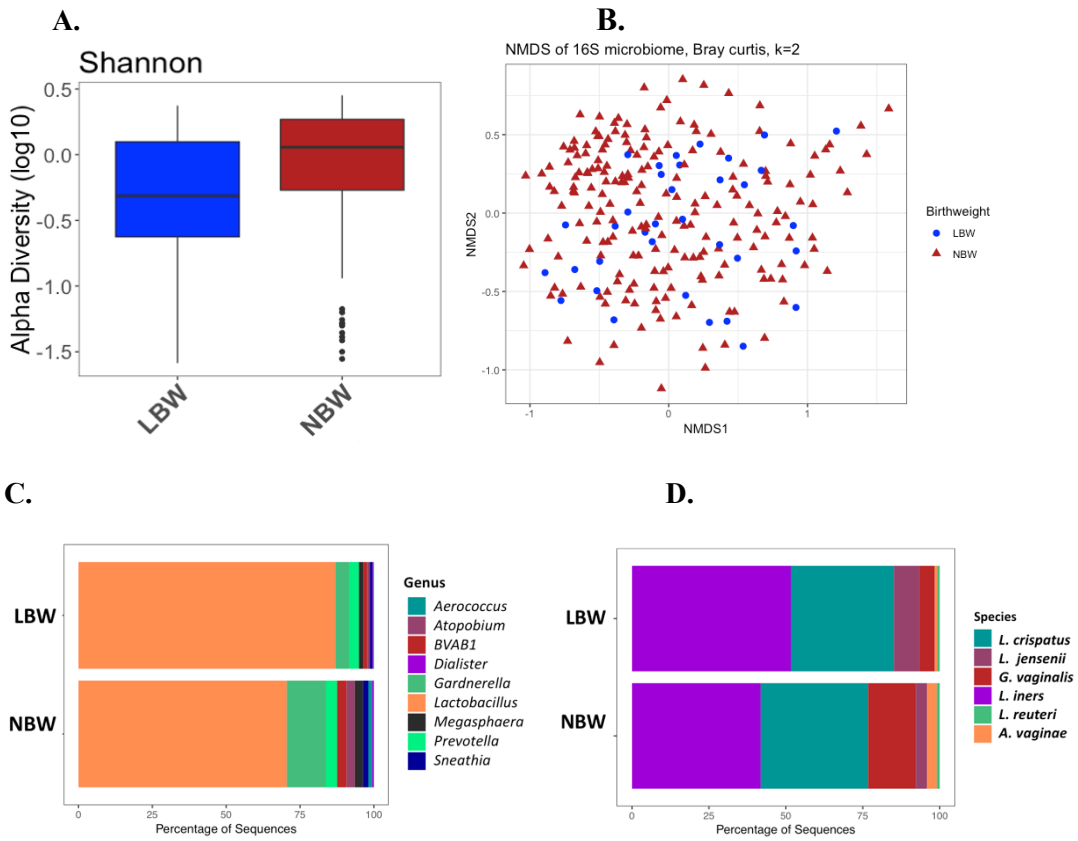


Figure 4.4 Diversity plot for LBW. **A.** Alpha diversity (Shannon; P=0.02) showing significant different within sample diversity and **B.** Bray Curtis (Adonis p=0.055) showing no difference in between sample diversity between women who delivered LBW and those who delivered normal birth weight babies. **C.** Bar plots showing relative abundant taxa at genus level by low birth weight. **D.** Bar plots showing relative abundant species by low birth weight.

MetagenomeSeq analysis was performed in order to identify taxa differentially abundant between women who delivered NBW infants compared to LBW deliveries. Thirteen OTUs that met threshold criteria of adj. p<0.05, absolute FC 1.25 and percentage presence in at least one group of 20 % were significantly different **(Figure 4.6).** Higher relative abundance of *L. coleohominis, Campylobacter,* [Mogibacteriaceae], *Suturella, A. minitum, V. dispar, N. gonorrhoeae, Jeotgalicoccus* were observed in women who experienced LBW deliveries while *Gardnerella, A. vaginae,* Coriobacteriaceae and Streptococcaceae were higher in women with NBW deliveries using taxa merged at the lowest taxonomic level. In addition to metagenomeSeq random forest model was used to predict taxa most influential of LBW but the predictive model did not produce reliable results as it had a sensitivity of <1%.

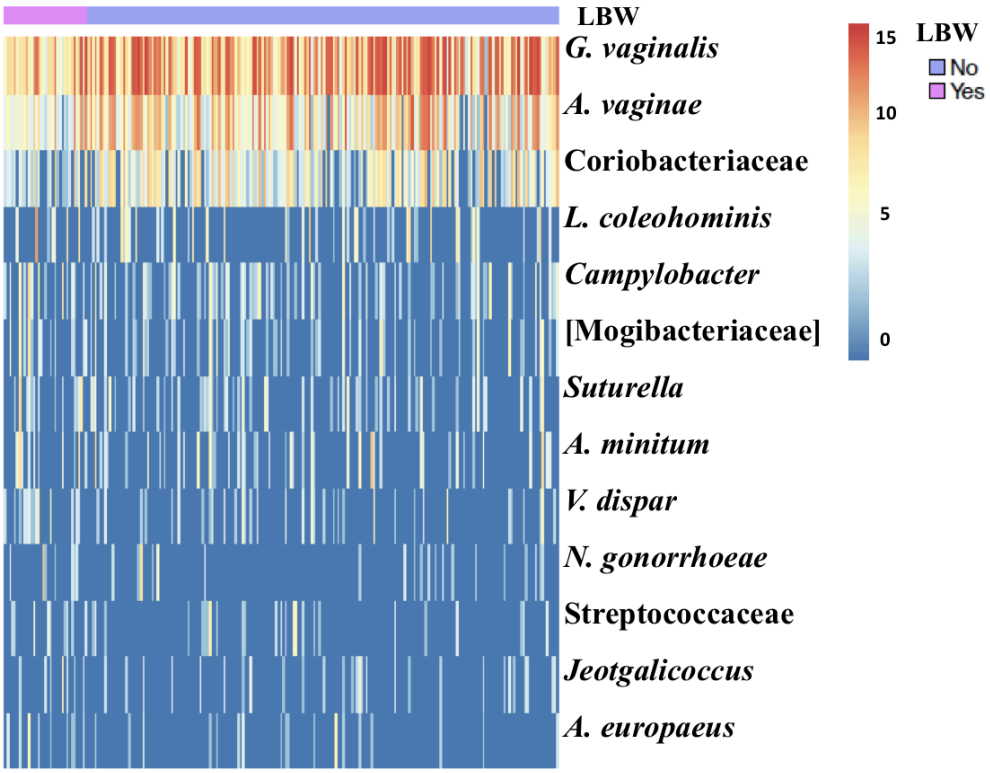


Figure 4.5 Heat map of differentially abundant taxa by LBW

Log_2 transformed and normalised counts using taxa merged at the lowest taxonomic level. Each column represents a woman and rows represent taxon. Red colour represents the most abundant while blue presents least abundant or absence of log_2 transformed OTU counts.

Using LEfSe at the genus level, *WAL_1855D* (p=0.015) and *Peptostreptococcus* (p=0.044) were higher in the group of women who delivered LBW babies with the effect size LDA score of >3.7 but their significance did not persist after FDR adjustment. On the other hand, *Gardnerella* (p=6e-04), *Mycoplasma* (p=0.015), Unclassified Coriobacteriaceae (p=0.04) and *Atopobium* (p=0.0053) were higher in women who delivered normal birth weight babies **(Figure 4.7A-C).** Only *Gardnerella* (p<0.001) persisted after FDR adjustment confirming the results obtained by MetagenomeSeq.

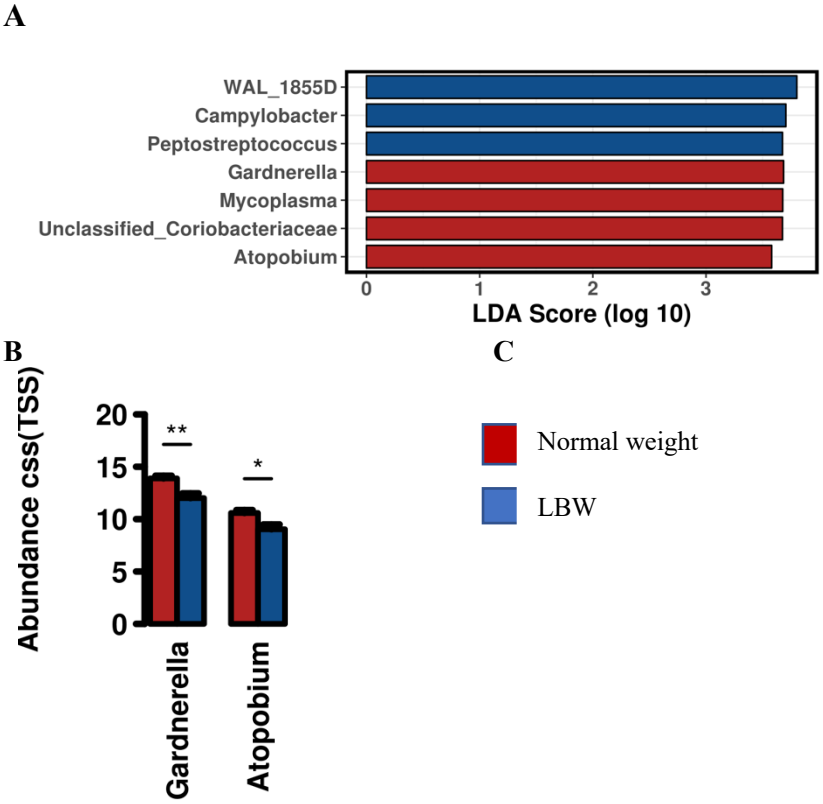


Figure 4. 6 A LEfSe showing significantly different taxa by LBW groups before FDR correction. The colors represent which group that taxa were found to be more abundant compared to the other group. **B.** Genus with significant different relative abundances. Community profiles were transformed using total sum counts (TSS) converting read counts to relative abundance then any bias introduced by TSS were corrected with cumulative-sum scaling (CSS). Standard error is depicted by error bars. Significantly different taxa are shown in a bar chart (p<0.05, Mann-Whitney-U test). Standard error is depicted by error bars. Group comparisons are done by Mann-Whitney-U test and annotated as *: p<0.05, **: p<0.01, ***: p<0.001.

4.3 Vaginal microbiota and small for gestational age (SGA) infants

Small for gestational age (SGA) is defined as infants below the 10th (or occasionally 3rd) centile of birthweight for their gestational age [389, 390]. These reference values are population-specific. For the purposes of this analysis, below 10th centile was calculated using an average weight of 3.200 kg at 40 weeks' gestation, derived from the weights of Angolan infants whose mean birth weight at 40 weeks' gestation was also 3.200kg [390]and since no published standard references for Zimbabwe were available.

Of the 233 women with complete data, 30 (13%) women experienced SGA deliveries while 203 (87%) had average for gestational age (AGA) deliveries. When stratifying characteristics by SGA, almost all variables including HIV status, BV, partner smoking status, maternal age, parity, and PIH had no significant association with SGA ($p>0.1$) Only GBS status and LBW had associations with SGA (both $p<0.05$) **(Table 4.5).** There were also observed trends towards more women with CST 1 (*L. iners*-dominated) vaginal microbiota delivering SGA and women with CST-3 (*G. vaginalis*-dominated) delivering AGA infants. In a multivariate logistic regression model, factors *a priori* considered as possible confounding factors (smoking partner, parity, HIV status, antibiotic use, gravida, and maternal age) were added to the model including GBS status and PIH which were significantly associated to SGA in univariate analysis. LBW could not be added due to its collinear effects with SGA as described above. Among these factors, GBS status and PIH were independently significantly associated with SGA ($p<0.05$) **(Table 4.6).** with a trend toward an association in maternal age where younger women were more likely to deliver SGA as compared to older women.

Table 4.5 Cohort characteristics by small for gestational age delivery

	AGA n=203	**SGA** n=30	**p- value**
Gravida, mean (SD)	3.0 (1.7)	2.7 (1.86)	0.412
Para, mean (SD)	1.4 (1.24)	1.2 (1.33)	0.533
Maternal age, mean (SD)	29.8 (6.38)	27.7 (6.81)	0.115
Gestational age at collection, mean (SD)	29.0 (6.53)	28.1 (5.34)	0.468
Partner smoker (%)	11 (5.4)	1 (3.3)	0.968
CST n (%)			0.101
1	53 (26.1)	13 (43.3)	
2	61 (30)	9 (30)	
3	89 (43.8)	8 (26.7)	
HIV-infected n (%)	23 (11.3)	5 (16.7)	0.590
Regimen n (%)			NaN
AZT 3TC	3 (14.3)	0 (0.0)	
TDF, FTC, EFV	18 (85.7)	5 (100.0)	
Blood CD4+ [cell/mm^3(n=24)] [median (IQR)]	488 (481-495)	450 (445-454.)	0.520
Nugent score, mean (SD)	3.2 (3.42)	2.7 (3.58)	0.488
BV n (%)			0.484
BV	50 (24.6)	5 (16.7)	
Intermediate	3 (16.7)	4 (13.3)	
Normal	119 (58.6)	21 (70)	
Vaginal douching n (%)	99 (48.8)	12 (40)	0.483
pH, mean (SD)	4.2 (0.68)	4.3 (0.76)	0.269
LBW n (%)	20 (9.9)	15 (50)	**<0.001**
Preterm n (%)	33 (16.3)	9 (30)	0.116
GBS positive n (%)	67 (33)	16 (53.3)	**0.049**
Pregnancy induced hypertension n (%)	10 (4.9)	4 (13.3)	0.162
Previous poor outcome n (%)	64 (31.5)	10 (33.3)	1.000
History of previous preterm delivery	29 (14.3)	6 (20.0)	**0.586**

LBW= low birth weight, GBS= group B streptococcus, BV=bacterial vaginosis,

CST= community state type, SGA= small for gestational age

Table 4.6 Multivariate logistic regression of factors associated with Small for gestational age

	OR	**95% CI**	**P values**
Gravida	1.21	0.84-1.70	0.29
Maternal age	0.92	0.84-1.01	0.08
Pregnancy induced hypertension	4.39	1.00-17.5	**0.04**
Partner smoker	0.4	0.02-2.59	0.42
GBS positive	2.52	1.09-5.96	**0.03**

Parity	1.85	0.46-7.39	0.38
HIV-infected	0.71	0.21-2.31	0.58
Antibiotic use	2.31	0.66-7.08	0.16

GBS= group B streptococcus. CI= confidence interval

Higher alpha diversity was observed within vaginal microbiota of women who delivered AGA infants as compared to SGA deliveries by Shannon (p= 0.02) and Simpson (p= 0.01) indices after post hoc (Dunn's) test adjustment **(Figure 4.8A).** Beta diversity measured using Bray Curtis distances revealed no significant differences between groups (p= 0.285) **(Figure 4.8B).** Similar, to the LBW analysis, bar plots of the most abundant genera showed *Lactobacillus* at higher relative abundance in the SGA samples while *Gardnerella* was relatively more abundant in women who delivered AGA infants. (**Figure 4.8C).** At the species level, *L. iners* was more abundant in mothers of SGA infants, and *A. vaginae* and *L. reuteri* was less abundant as compared to AGA. However, statistics was not performed on these comparisons.

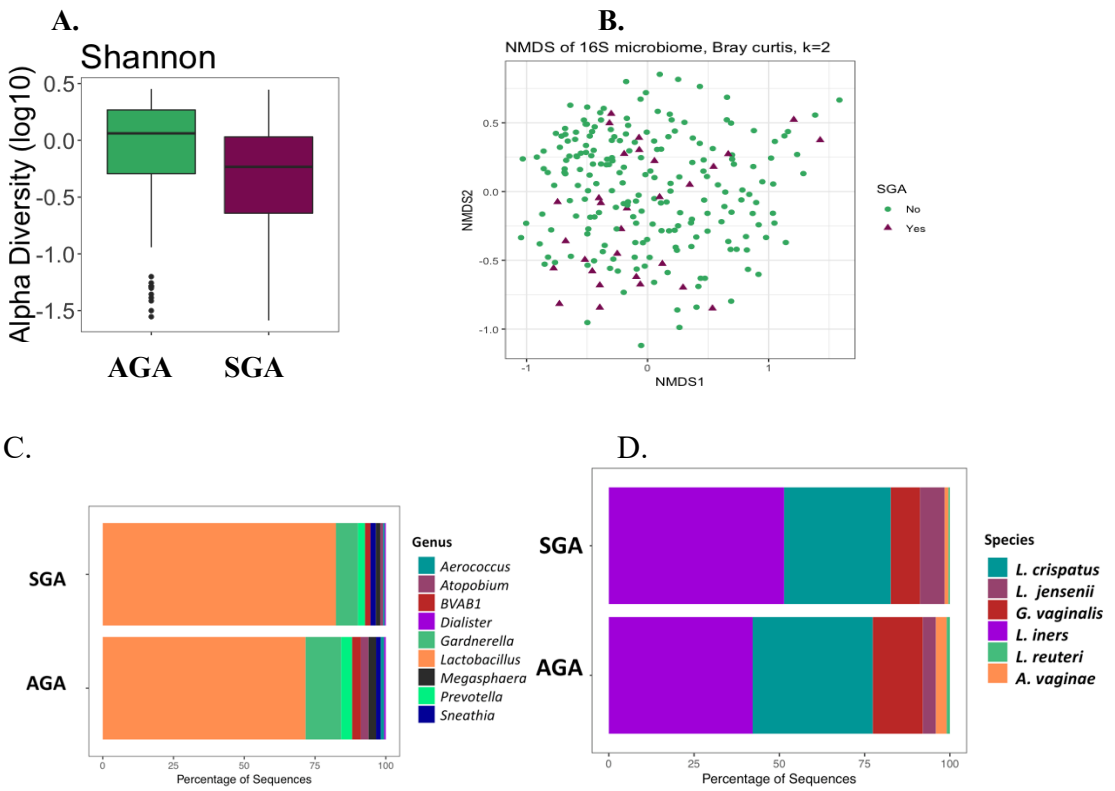


Figure 4.7 A. Alpha diversity in SGA versus AGA (p=0.02) **B.** Beta diversity (Adonis p=0.285). **C.** Bar plots showing relative abundant taxa at genus level by small for gestational age. **D.** Bar plots showing relative abundant species by small for gestational age. AGA=average gestational age SGA =small for gestational age.

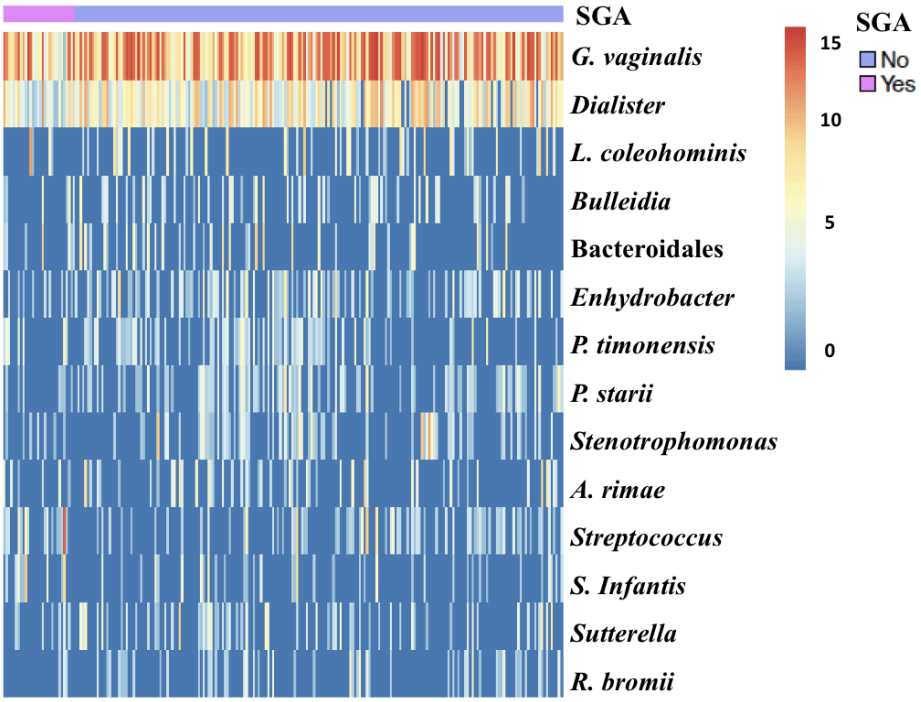


Figure 4.8 Heat map of results from MetagenomeSeq showing differentially abundant genera by SGA deliveries. Differential abundance testing was performed on taxa merged at the lowest taxonomic level. Each column represents a woman and rows represent taxa. Red colour represents the most abundant while blue presents least abundant or absence of $\log_2$ transformed OTU counts.

Using LEfSe, there were three genera whose relative abundance was significantly higher in SGA deliveries namely *Prevotella* (p=0.042), *BVAB-1* (p=0.005) and *Atopobium* (p=0.007) with LDA scores > 2.99, but none persisted after FDR adjustment. On the other hand no genera had statistically significantly higher relative abundance in AGA deliveries **(Figure 4.10).**

A.

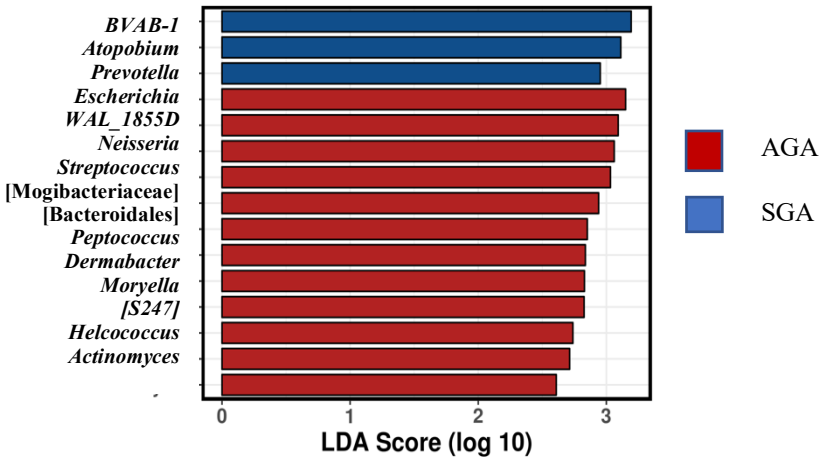


Figure 4.9 LefSe showing significantly different taxa by SGA groups. The colors represent which group that taxa were found to be more abundant compared to the other group. Community profiles were transformed using total sum scaling (TSS) converting read counts to relative abundance. Significantly different vaginal taxa are shown as bar chart ($p<0.05$, Mann-Whitney-U test). Standard error is depicted by error bars. Group comparisons are done by Mann-Whitney-U test and annotated as *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

4.4 Discussion

Our study sought to identify an association between vaginal microbiota and birth outcomes such as preterm delivery, SGA and LBW, in Zimbabwean pregnant women. We did not find any strong associations between poor birth outcomes and vaginal microbiota. However, some associations were observed between birth outcomes and specific taxa, including *L. iners*. In line with our findings, other studies have reported the associations between *L. iners* and preterm deliveries, while in some, *L. crispatus* was denoted to be protective of poor outcomes [150, 391]. Interestingly, regardless of which outcome we assessed, high *G. vaginalis* relative abundance was seemingly associated with good birth outcomes.

It has been demonstrated that BV and specific vaginal anaerobes are associated with poor birth outcomes in various ethnic groups, Nigeria [18], USA [16], India [47] and other areas [155]. This however was not the case with our findings. We found no association between BV and preterm, SGA nor LBW deliveries. Indeed, we observed trends that contrasts with published dogma, showing more BV negative women experiencing SGA deliveries and having a lower chance of delivering at term in this cohort.

A decrease in richness and diversity has been observed in healthy pregnancy as compared to non-pregnant women [6], and a significant decrease in richness and diversity between the first and second trimesters has been associated with preterm delivery [48]. This agrees with our study findings, where we observed lower microbial diversity within vaginas of women who experienced SGA and LBW (significantly different; alpha diversity; Shannon) as compared to normal deliveries, although we did not follow women longitudinally therefore do not know whether this was a decrease in diversity as in Stout *et al.,* (2017). When considering beta diversity, Subramaniam and colleagues did not find statistically significant differences between vaginal communities of pregnant women who delivered preterm infants and those who delivered term infants [46], concurring with our study findings. In fact, no significant differences in beta diversity measures were observed in all birth outcomes in question (SGA, LBW, PTB). Although the authors attributed it to small sample size and perhaps dynamic changes of the vaginal microbiota [46], our study had a much larger sample size yet had concurring findings.

In our observations, we did not find evidence of significant associations between CSTs and poor outcomes. There were more women in CST1 (*L. iners*-dominated) who had LBW and SGA deliveries while on the other hand more women with NBW and AGA deliveries were found in CST3 (*G. vaginalis*-dominated).

In this Zimbabwean cohort, older age, being multigravida and/ or multiparous were associated with having PTB. Many prior studies have shown that poor outcomes were positively correlated with older age, including some in African women [387, 392-394]. However, in our study, young maternal age was associated with having an SGA and LBW delivery. This is also in agreement with some other prior studies [395-398]. Likewise, having a history of a previous poor pregnancy outcome was independently associated with LBW, even after adjustment of possible confounders. These findings agree with studies conducted in northern Tanzania and Australia which reported the association of previous poor outcomes with subsequent PTB and LBW deliveries [399, 400]. This points to the fact that many factors affecting risk for poor birth outcomes may be host-determined. In line with other studies, our study demonstrates that women who had PIH were highly likely to deliver LBW, preterm and SGA infants as compared to the normal deliveries. PIH has been unanimously associated with poor outcomes in all ethnic groups and geographical locations such as Zimbabwe [401], India [402] and Brazil [403], to name a few. In some settings, women with PIH may be more likely to deliver premature or LBW infants because of induction of labour or cesarean sections, however this only occurs in the case of emergency in Zimbabwe.

We observed that women with vaginal GBS colonisation were more likely to have an SGA infant as compared to GBS negative women. Most previous data that suggest associations of GBS with premature birth, stillbirths but rarely SGA infants [298, 404]. Therefore, this association could be explored in future studies.

HIV infection has been reported as a known risk factor for adverse birth outcomes [192]. HIV status was associated with PTB and LBW in our study in agreement with various studies where HIV status is highly associated with adverse pregnancy outcomes [181, 187]. Since all the women in our cohort were on antiretrovirals, it is difficult to ascertain whether it was HIV infection, or the drugs used to treat it that could be causing PTB and LBW. In prior studies it has been reported that some regimens such as Protease inhibitors (PIs) [192] and AZT [193] were associated with PTB and LBW and that

generally HAART users were more at risk of poor pregnancy outcomes as compared to non-users [187, 188]. However, other studies found no significant associations between different regimens (NNRTI, PI, NRTI) and poor birth outcomes [194, 195]. In the days of Option B+, it will be difficult to tease apart whether HIV infection or ART contribute to poor outcomes, but studies on different regimens are warranted.

In summary, from the findings of our study it is possible to conclude that factors influencing pregnancy outcomes are multifactorial, and vaginal microbiota may play a role. *L. iners* was associated with some poor birth outcomes while *G. vaginalis* was associated with good birth outcomes. Furthermore, there was no strong influence of BV status or CST observed on pregnancy outcomes, except for lack of BV which was associated with LBW. However, a longitudinal study would be ideal in future to further delineate these relationships considering limitations in our study. Microbiota samples were not collected at the same time point in all women and bias may have been introduced by lack of data on pregnancy outcomes on some women.

Chapter 5. Vaginal microbiota and cytokines during pregnancy

Most adverse reproductive health outcomes are likely mediated by inflammatory processes that disrupt maternal fetal tolerance [405-409]. Successful pregnancies have been associated with the immunological switch from Th1 (T helper 1) to Th2 (T helper 2) cytokines [334]. Th2 cytokines have been suggested to play an important role in successful pregnancies, while Th1 cytokines are deleterious to pregnancies [334, 405, 407]. The Mother's immune system should tolerate the development of the "foreign foetus" [407]. However, a balance between immunosuppression in protection of the foetus and maintaining immunological functions to protect against infectious diseases is necessary [407]. Proinflammatory cytokines, such as IL-1β, IL-6, IL-8, have been found to be increased in various tissues in women who experience poor pregnancy outcomes such as pregnancy loss, spontaneous preterm labour and PTB [318, 337, 408, 410].

As shown in previous studies, vaginal microbiota are known to influence the cytokine profile in the female reproductive tract [116], while it has further been suggested that proinflammatory cytokines may in turn serve as regulators of vaginal microbiota [411]. Certain proinflammatory (IL-1β, IL-6, IL-8) cytokines can promote the growth of *Lactobacillus* at lower concentrations, while at high concentrations these cytokines can stimulate the growth of opportunistic pathogens [411]. The relationship between vaginal microbiota and cytokine levels in the vagina during pregnancy has not been established.

Luminex was used to measure the concentrations of 27 cytokines in vaginal swabs. This analysis is based on 324 women selected by HIV and BV status information. Not all samples were analysed due to limitation of resources. Of the 27 cytokines measured, IL-12p70, IL-15, IL-10, IL-5, G-CSF and IFN-γ did not pass quality control procedures, and these cytokines were thus excluded **(Appendix A).** Therefore, this analysis is based on 20 cytokines for which high quality data was generated (**Figure 5.1**). These cytokines were grouped according to primary biological functions, namely adaptive (IL-2, IL-13, IL-4, IL-17A), regulatory (IL-10, IL-1ra), growth factors (IL-7, IL-9, FGF-basic, PDGF-BB, VEGF, GM-CSF), chemokines (IL-8, Eotaxin, IP-10, MCP-1,

MIP-1α, MIP-1β, RANTES) and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) **(Figure 5.1).**

Women were grouped into three categories based on the concentrations of inflammatory cytokines and chemokines (IL-1β, IL-6, TNFα, IL-8, Eotaxin, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES) in their genital tracts by factor analysis as described in Masson *et al.,* (2015). Briefly factor scores of grouped cytokines were generated in stata, with women grouped as having high inflammation if their inflammatory factor score was in the upper quartile (≥ 75th percentile), medium inflammation if their score was in the interquartile range (<75th - >25th percentile) and low inflammation if their score was in the lower quartile (≤25th percentile. Three community state types (CSTs) where identified; CST1: *L. iners* dominated, CST2: *L. crispatus* dominated, CST3: *G. vaginalis* dominated as described in chapter 3. The relationships between cytokines, cytokine functional groups and genital inflammation and vaginal microbiota and birth outcomes were then investigated.

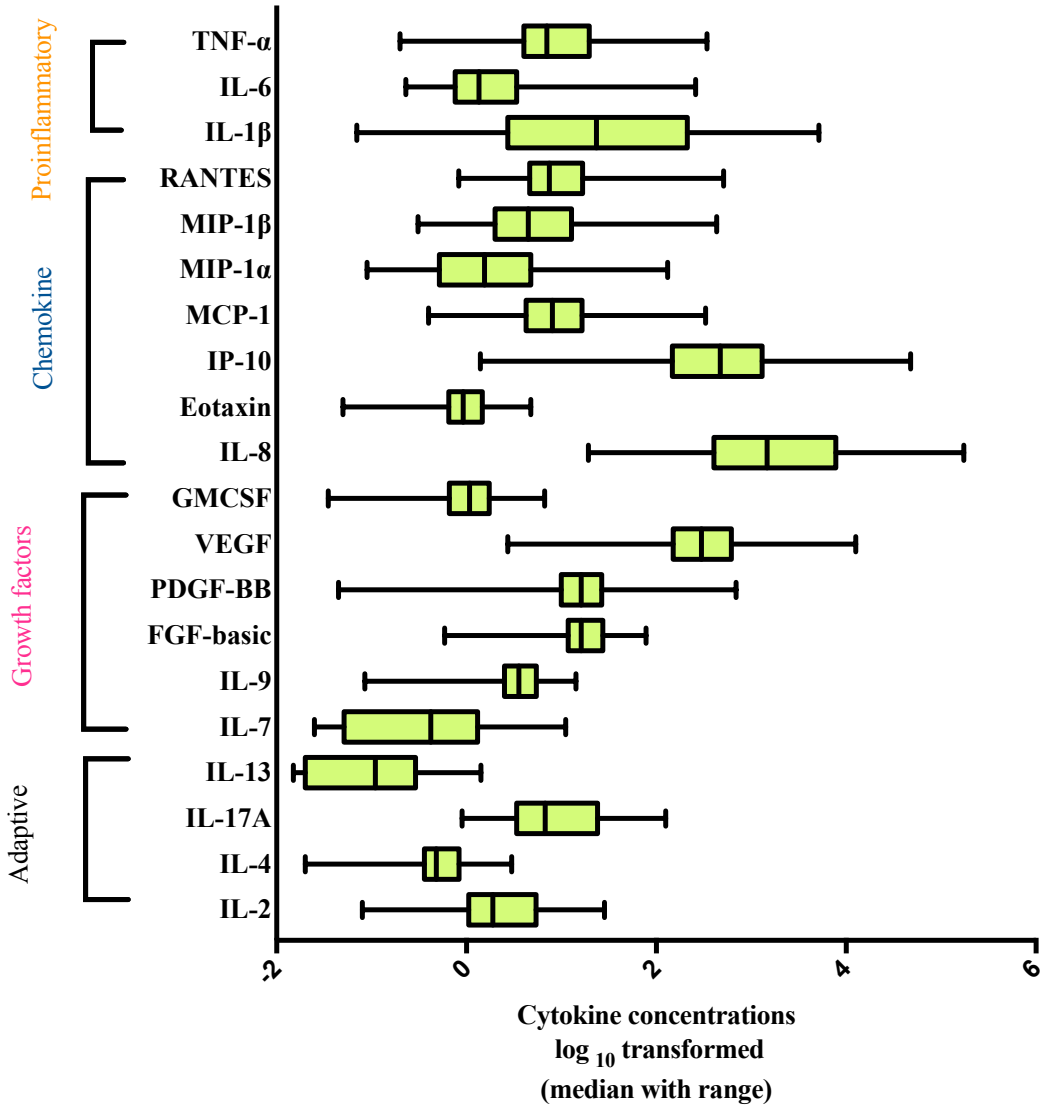


Figure 5.1 The overall concentrations of cytokines in vaginal secretions of 324 women. Log_{10}-transformed, cytokine concentrations in pg/ml are depicted by box and whisker plots indicating the median concentrations (middle line), 25th (left) and 75th (right) percentiles and ranges (whiskers) of cytokine concentrations. Four functional groups of cytokines are represented on the left side.

5.1 Relationship between cytokines and the vaginal microbiota during pregnancy

The relationships between genital inflammation, demographic and clinical factors and the microbiota were investigated using Mann Whitney U test and Chi-squared test. It was found that maternal age, Nugent score (BV), parity, and CST were each significantly associated with inflammation ($p<0.05$) **(Table 5.1).**

Table 5.1 Characteristics of the cohort by level of vaginal inflammation

	High inflammation (n=81)	**Medium inflammation (n=164)**	**Low inflammation (n=79)**	**p-value**
Partner smoker [n (%)]	5 (6.2)	14 (8.5)	3 (3.8)	0.376
Antibiotics use past 3 months [n (%)]	10 (12.3)	21 (12.8)	8 (10.1)	0.831
SGA current pregnancy [n (%)]	4 (8.2)	20 (18.0)	4 (6.7)	0.058
Preterm [n (%)]	7 (13.7)	22 (18.5)	13 (21.3)	0.58
Still birth [n (%)]	3 (5.4)	7 (5.6)	2 (3.2)	0.762
LBW [n (%)]	4 (8.2)	19 (17.1)	12 (20.0)	0.216
Gestational age at collection in weeks, mean (SD)	29.0 (5.74)	28.5 (6.98)	27.7 (5.62)	0.388
Vaginal douching [n (%)]	41 (50.6)	80 (48.8)	40 (50.6)	0.946
BV Nugent score [mean (SD)]	5.5 (3.41)	3.3 (3.33)	1.3 (2.54)	**<0.001**
GBS [n (%)]	45 (40.9)	42 (41.2)	52 (36.1)	0.645
BV (%)				**<0.001**
BV	43 (53.1)	6 (7.6)	37 (22.6)	
Intermediate	14 (17.3)	6 (7.6)	36 (22.0)	
Normal	24 (29.6)	67 (84.8)	91 (55.5)	
pH, mean (SD)	4.2 (0.65)	4.2 (0.70)	4.2 (0.76)	0.711
Gravida, mean (SD)	2.6 (1.39)	3.0 (1.84)	2.8 (1.54)	0.14
Para, mean (SD)	1.1 (1.04)	1.5 (1.36)	1.4 (1.02)	**0.024**
Maternal age in years, mean (SD)	27.66(6.21)	29.3 (6.04)	30.4 (6.96)	**0.025**
CST [n (%)]				**0.019**
1	28 (34.6)	52 (31.7)	19 (24.1)	
2	16 (19.8)	39 (23.8)	33 (41.8)	
3	37 (45.7)	73 (44.5)	27 (34.2)	
Pregnancy induced hypertension [n (%)]	3 (3.7)	13 (7.9)	4 (5.1)	0.388
Previous poor outcome [n (%)]	22 (27.2)	52 (31.7)	28 (35.4)	0.527
HIV [n (%)]	11 (13.6)	24 (14.6)	7 (8.9)	0.447

LBW= low birth weight, GBS= group B streptococcus. BV=bacterial vaginosis, CST= community state type.
SGA= small for gestational age, SD=standard deviation

We performed a multivariate model, considering the factors that may influence genital inflammation. The factors included HIV, vaginal douching, BV, maternal age and parity (BV was not included due to its collinearity with CST). CST2 was used as a reference here, CST3 and CST1 and maternal age were independently associated with inflammation (p=0.008, p=0.02 and p<0.002, respectively; **Table 5.2).**

Table 5.2 Multivariate analysis of factors associated with inflammation

	OR	**95% CI**	**p- value**
Maternal age	1.11	1.04-1.18	0.002
Vagina douching	1.41	0.62-3.29	0.42
CST3	2.83	(1.30, 6.14)	**0.008**
CST1	3.04	(1.32, 7.00)	**0.02**
HIV	1.62	(0.59, 4.41)	0.35
Parity	0.69	0.45-0.96	**0.078**

BV= bacterial vaginosis, CI= confidence interval, OR= odds ratio

We then went on to analyse vaginal microbiota of women in high versus low genital inflammation groups, using RF and metagenomeSeq in order to identify taxa associated with inflammation. Twenty-two taxa were significantly different between women with high versus low inflammation by metagenomeSeq (adj. $p < 0.05$, absolute FC > 1.25 and percentage presence in at least one group of 20%) **(Figure 5.3).** *Aerococcus, A. vaginae, G. vaginalis, Dialister, Megasphaera, Gemella, Mycoplasma, Prevotella timonensis* and *L. iners* among others were associated with high inflammation. Conversely, some *Lactobacillus* species including *L. crispatus* and *L. reuterii,* and *Staphylococcus* and *Roseburia* were significantly associated with low inflammation. RF replicated some taxa produced by metagenomSeq, taxa such as *L. crispatus, Megasphaera, Dialister, Prevotella, G. vaginalis, L. iners, L. reuteri, A vaginae, Aerococcus and* BVAB1 were the most highly influential taxa for predicting inflammation with mean decrease in Gini score >3 by RF. **(Figure 5.2B).** The model displayed the top 20 taxa for predicting inflammation in the test set, with an OBB error rate of 25% with a positive predictive value of 71%; negative predictive value of 72% and a degree of accuracy of 71%.

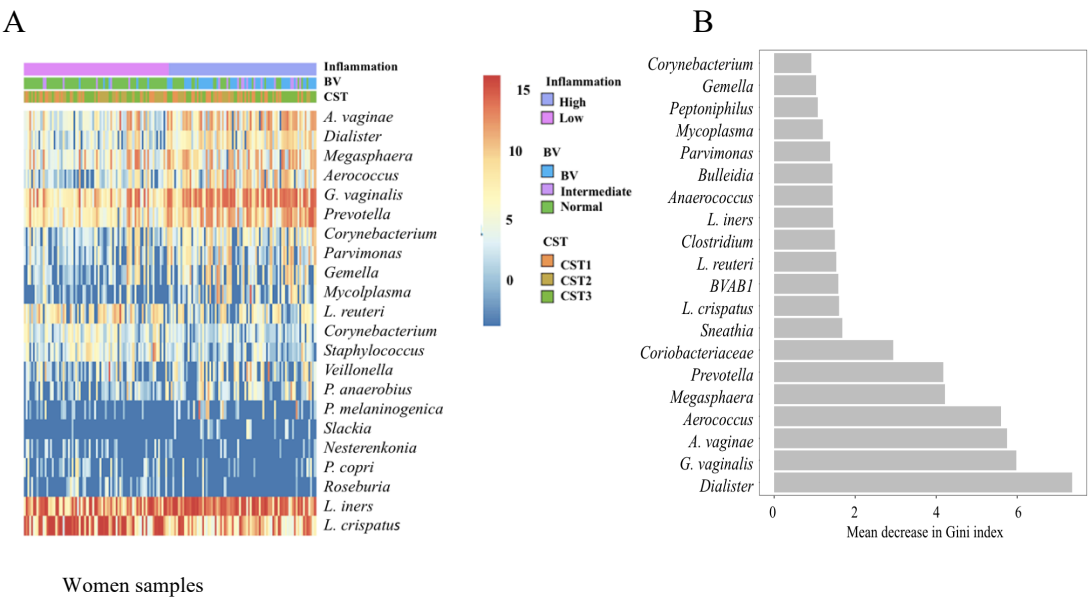


Figure 5.2 Taxa most predictive of inflammation using taxa merged at the lowest taxonomic level. **A** Supervised hierarchical clustering (Bray Curtis distances) of differentially abundant taxa in women with and without genital inflammation determined using metagenomeSeq. The heatmap shows $\log_2$-transformed standardized counts. Each column represents a woman and rows represent taxa. **B** Most important taxa associated with inflammation identified using random forest. The X-axis shows the mean decrease in the Gini index (length of bars represent relative predictive ability of each taxon).

Further analysis was done to find the direction of relationship between taxa identified as predictive of genital inflammation by random forest using Mann Whitney U test for independent samples and Welch Two Sample t-test of unequal variance to show the significant differences between the mean relative abundance of taxa in inflammation groups. *G. vaginalis, Dialister, A. vaginae, Aerococcus, Prevotella* ($p<0.001$), *L. iners, Megasphaera*, *BVAB1*($p<0.05$) had significantly higher means in women with high inflammation while *Staphylococcus* ($p<0.001$) and *L. crispatus (*$p <0.007$ were significantly higher in women with low inflammation **(Figure 5.3).** It is interesting to note that *L. iners* was positively associated with inflammation which concurs with its

association with intermediate and BV+ status as described in the previous chapter.

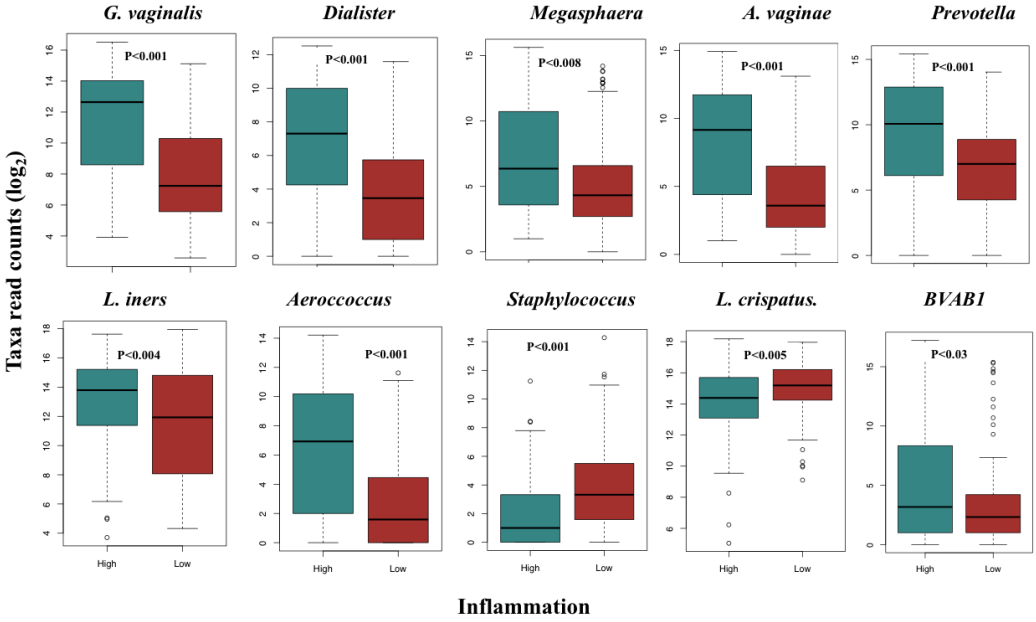


Figure 5.3 Summaries of median differences of taxa counts most predictive of inflammation (log_2 counts). Relationships are depicted by box and whisker plots indicating the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of the group cytokine concentrations (log_{10}-transformed (pg/ml). Mann Whitney U for independent samples was used to show the significant differences between the mean relative abundance of taxa in inflammation groups. All p-values were adjusted for multiple comparisons using the false discovery rate (FDR) step-down approach (Columb and Sagadai, 2006).

5.2 Cytokine concentrations by BV status

One-way ANOVA was used to evaluate the relationships between log_{10}-transformed vaginal cytokine concentrations and BV status. Pairwise comparisons by BV status showed significant differences between cytokine concentrations in women with BV (Nugent score: 7-10) or intermediate Nugent scores (Nugent score: 4-6) as compared to BV- women (Nugent score: 0-3), while few cytokines were significantly different between women with intermediate Nugent scores and BV+ women **(Figure 5.4).** Most of the cytokine concentrations followed the same pattern of being upregulated in the vaginal fluid of BV+ women followed by intermediate then BV- **(Figure 5.4; Figure 5.5).** However, IP-10 was significantly downregulated in BV+ women as compared to women with intermediate and healthy microbiota, while concentrations were similar in intermediate and BV- women. The concentration of VEGF was lower in all women with intermediate microbiota as compared to BV+ and BV- groups. **(Figure 5.5)**.

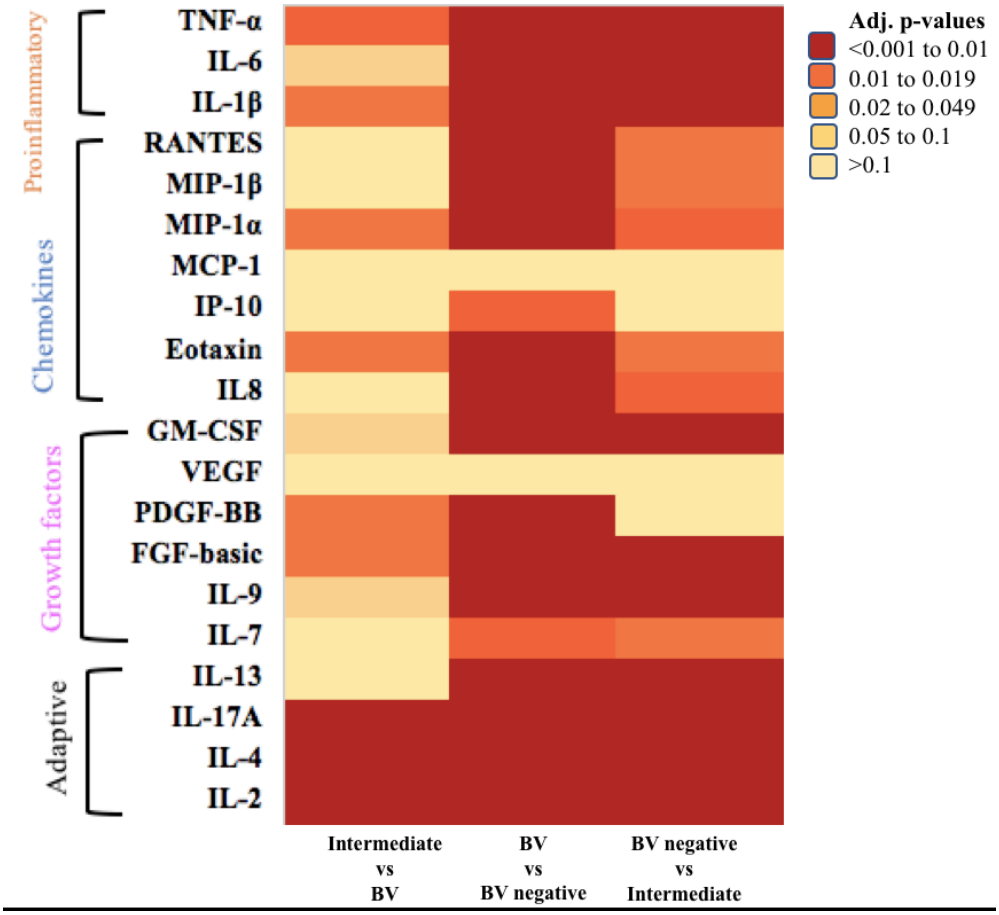


Figure 5.4 Heatmap summary of significantly different cytokines by BV status (BV, Intermediate and BV negative) using log_{10}-transformed (pg/ml) vaginal cytokines concentrations. All significant p-values <0.05 are represented by red shades of colour, with deep red being the lowest p-value while all p-values >0.05 are represented but yellow/brown shades of colour with yellow being the highest p value. Significance testing was done using one-way analysis for variance (ANOVA) and Tukey Honest Significant Differences post hoc test for pairwise comparisons. All p-values were adjusted for multiple comparisons using the false discovery rate (FDR) step-down approach (Columb and Sagadai, 2006).

Analysis of possible associations between individual cytokine concentrations by BV status were performed. Individual cytokine concentrations were significantly different between BV+, Intermediate and BV- (negative) women with p<0.001 except for VEGF and MCP-1 which were not significantly different p>0.05 in all the three groups (**Figure 5.5)** IP-10 was lower in the BV+ women as compared to intermediate and BV- women

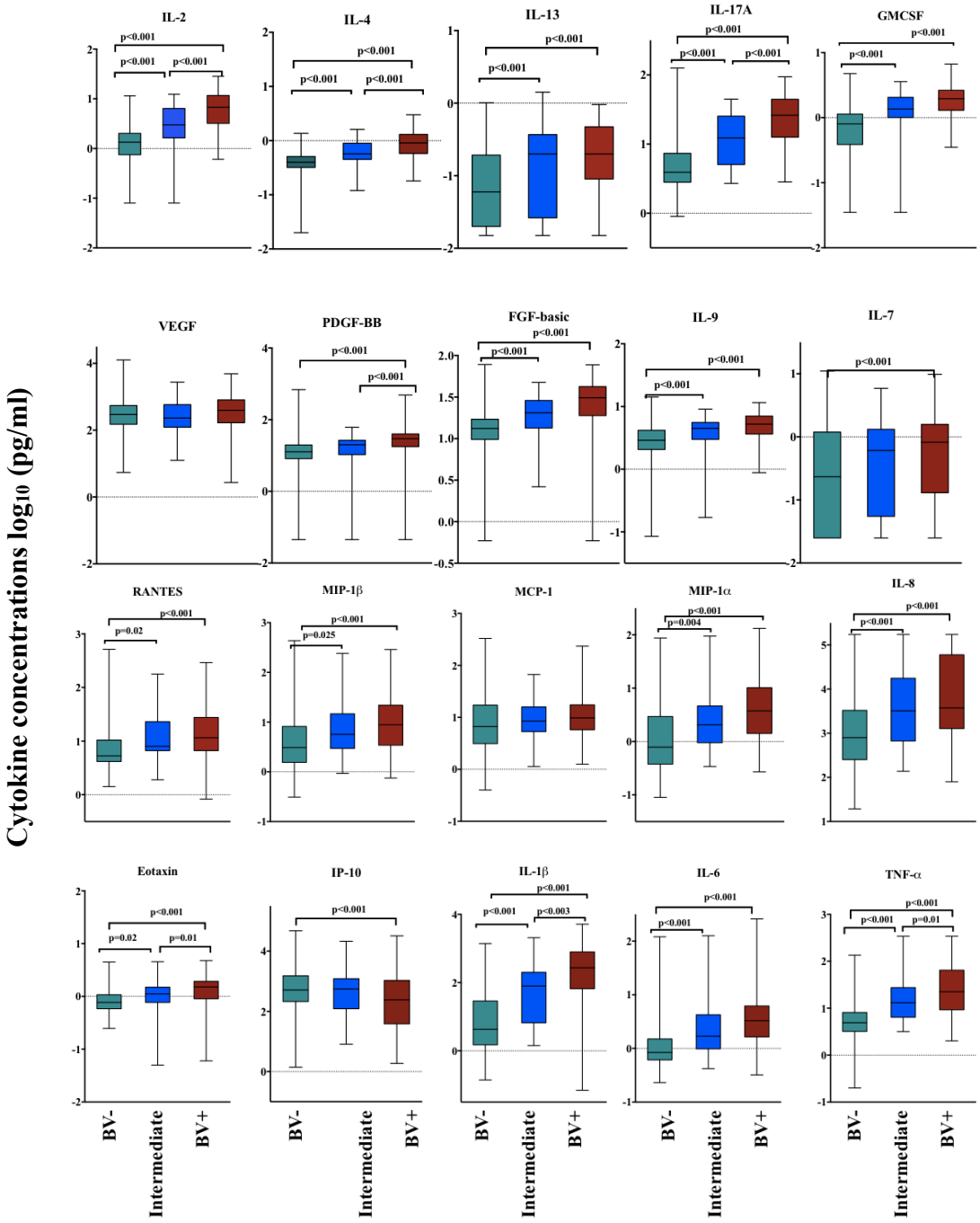


Figure 5.5 Vaginal cytokines concentrations by BV status. Relationships are depicted by box and whisker plots indicating the medians (middle line), 25th (bottom line) of boxes and 75th percentiles (top line), and the ranges (whiskers) of the log_{10}-transformed (pg/ml) cytokine concentrations by BV status. Non-parametric assessments of variation between groups were carried out through the Kruskal-Wallis Analysis of Variance (ANOVA) and Tukey Honest Significant Differences post hoc test being applied to test for the pairwise comparisons. All p-values were adjusted for multiple comparisons using the false discovery rate (FDR) step-down approach (Columb and Sagadai, 2006).

Cytokines were grouped according to primary biological functions and factor scores (representative of each cytokine within each group) were generated for each functional group using factor analysis. The factor scores for all of the functional groups were highest in women with BV followed by those with intermediate microbiota and then BV- (normal) women. **(Figure 5.6)**

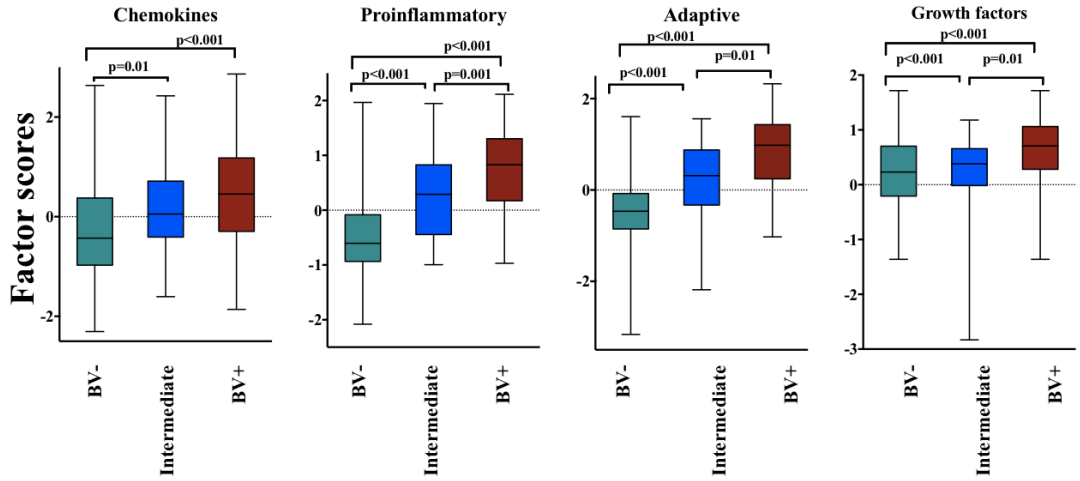


Figure 5.6 Cytokine functional factor scores by BV status. Relationships are depicted by box and whisker plots indicating the medians (middle line), 25th (bottom line) and 75th percentiles (top line), and the ranges (whiskers) of the cytokine factors for each group by BV status. Non-parametric assessments of variation between groups was carried out through the Kruskal-Wallis Analysis of Variance (ANOVA) and Tukey Honest Significant Differences post hoc test being applied to test for the pairwise comparisons. All p-values were adjusted for multiple comparisons using the false discovery rate (FDR) step-down approach (Columb and Sagadai, 2006).

5.3 Community state type and cytokine concentrations

One way ANOVA was used to identify significant differences between $\log_{10}$-transformed cytokine concentrations and CST, followed by a Tukey Honest Significant Differences post hoc test (Tukey HSD) for performing multiple pairwise-comparisons between the medians of groups**.** Multiple cytokines differed significantly between women with different CSTs (adjusted $p<0.05$): 15/20 for CST3 vs CST2, 9/20 for CST3 vs CST1 and 4/20 for CST2 vs CST1 **(Figure 5.7).**

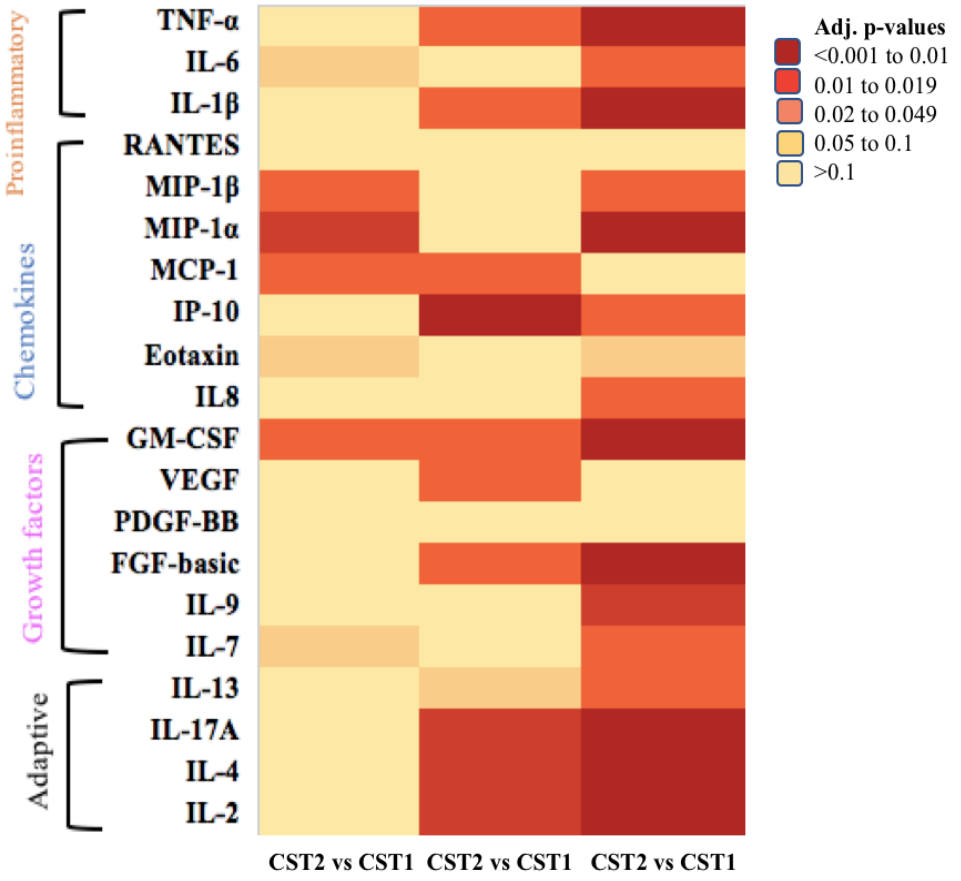


Figure 5.7 Heatmap summary of cytokine associations with microbial composition highlighting cytokines significantly different by community state type (CST1, CST2 and CST3). All significant p-values <0.05 are represented by red shades of colour, deep red being the lowest p-value and the most significant while all p-values ≥0.05 are represented but yellow /brown shades of colour with yellow being the highest p value. Significance testing was done using one-way analysis of variance (ANOVA) and Tukey Honest Significant Differences post hoc test for pairwise comparisons. All p-values were adjusted for multiple comparisons using the false discovery rate (FDR) step-down approach (Columb and Sagadai, 2006).

GM-CSF, TNF-α, IL-1β, FGF-basic, IL-17A, IL-4, IL-2, IL-13, IL-7, IL-9, PDGF-BB, IL-8, MIP-1α, MIP-1β, Eotaxin, RANTES and IL-6, were all significantly higher in CST1 and /or CST3 as compared to CST2 while IP-10 was downregulated in CST3 as compared to CST1 and CST2 (**Figure 5.8).**

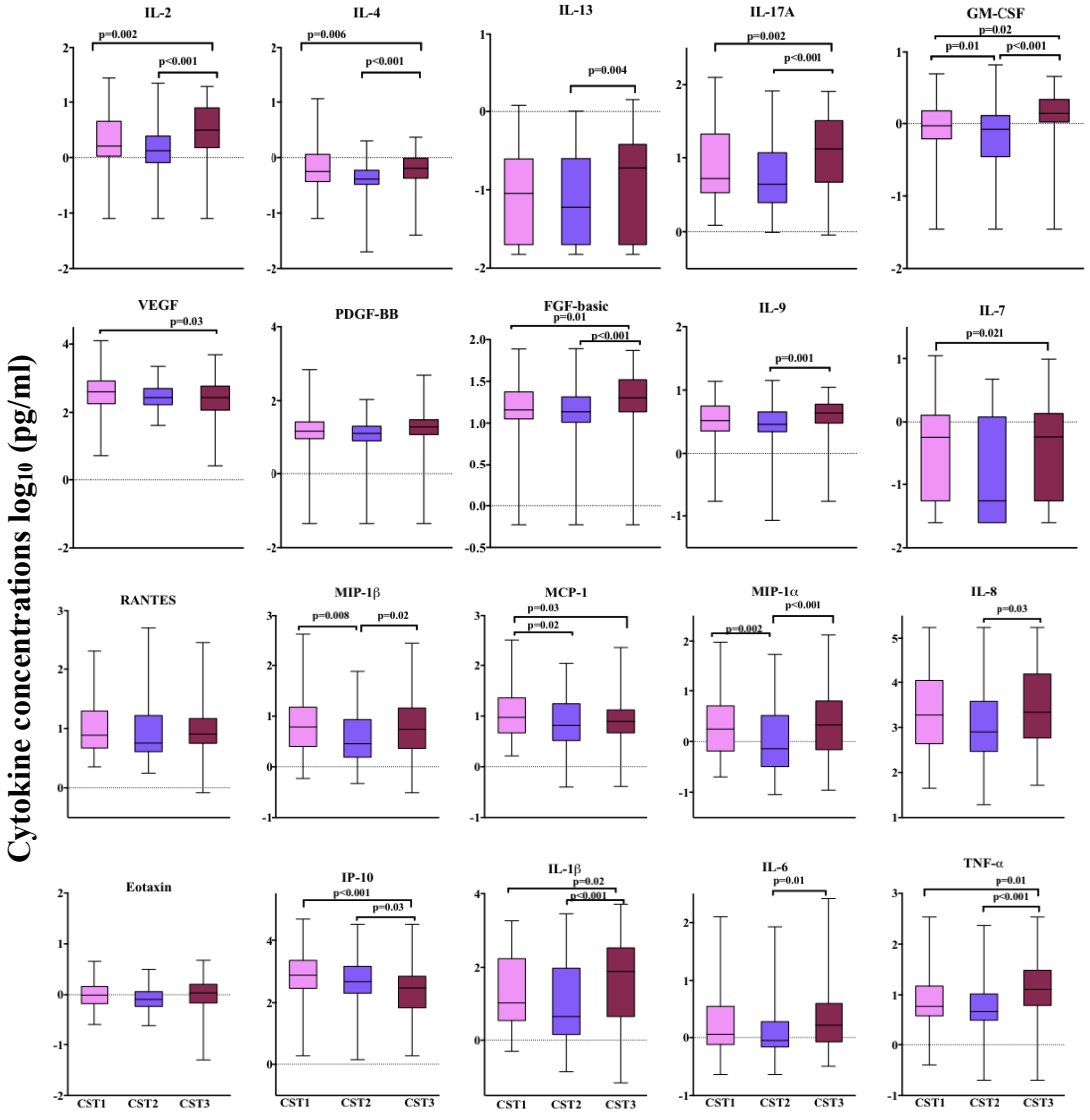


Figure 5.8 Vaginal cytokine by community state type. Relationship are depicted by box and whisker plots indicating the medians (middle line), 25th (bottom line) of boxes and 75th percentiles (top line), and the ranges (whiskers) of the log_{10}-transformed (pg/ml) cytokine concentrations by community state type. Non-parametric assessments of variation between groups were carried out through the Kruskal-Wallis Analysis of Variance (ANOVA) and Tukey Honest Significant Differences post hoc test being applied to test for the pairwise comparisons. All p-values were adjusted for multiple comparisons using the false discovery rate (FDR) step-down approach (Columb and Sagadai, 2006).

Cytokines were grouped according to primary biological functions and factor scores were generated for each functional group using factor analysis. As expected the factor scores for proinflammatory, adaptive and growth factors were significantly higher in women with CST3 (*G. vaginalis*-dominated) as compared to CST1 (*L. iners*-dominated) and CST2 (*L. crispatus*-dominated) **(Figure 5.9).**

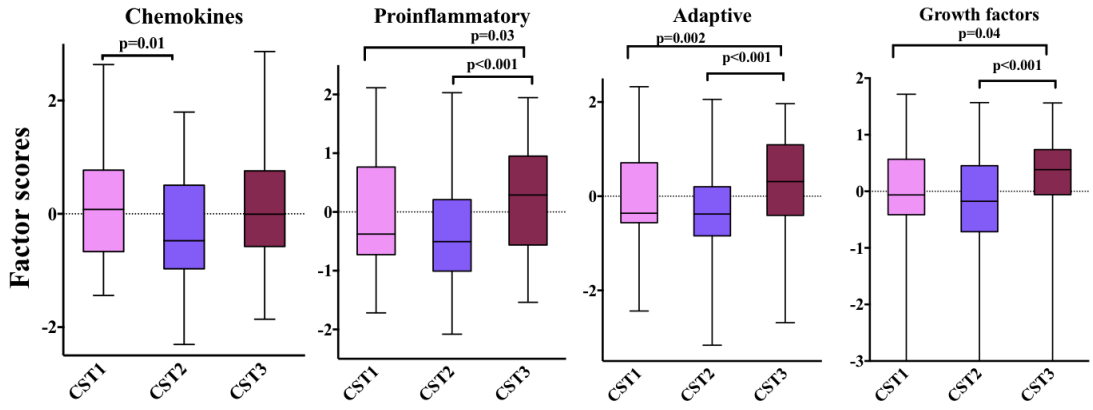


Figure 5.9 Cytokines functional factors score by community state type. Relationship are depicted by box and whisker plots indicating the medians (middle line), 25th (bottom line) and 75th percentiles (top line), and the ranges (whiskers) of the cytokine factor scores for each group by community state type. Non-parametric assessments of variation between groups was carried out through the Kruskal-Wallis Analysis of Variance (ANOVA) and Tukey Honest Significant Differences post hoc test being applied to test for the pairwise comparisons. All p-values were adjusted for multiple comparisons using the false discovery rate (FDR) step-down approach (Columb and Sagadai, 2006).

The relative abundance of eight taxa were significantly associated with one or more cytokines after adjustment for multiple comparisons ($R \geq 0.3$). Most of these eight taxa ($\geq 7/8$) correlated significantly with IL-2, IL-4 and IL-17A (adaptive), IL-1β and TNF-α (pro-inflammatory), and FGF-basic and GM-CSF (growth factors). *Gardnerella* relative abundance was most strongly positively correlated with cytokine concentrations, while *Lactobacillus* was negatively correlated with 16/20 of the cytokines (Figure 5.11A). Interestingly, IP-10 was the only cytokine that was inversely associated with dysbiotic bacteria, including *Megasphaera, Coriobacteriaceae*, *Dialister, Prevotella* and *Gardnerella* **(Figure 5.10A).**

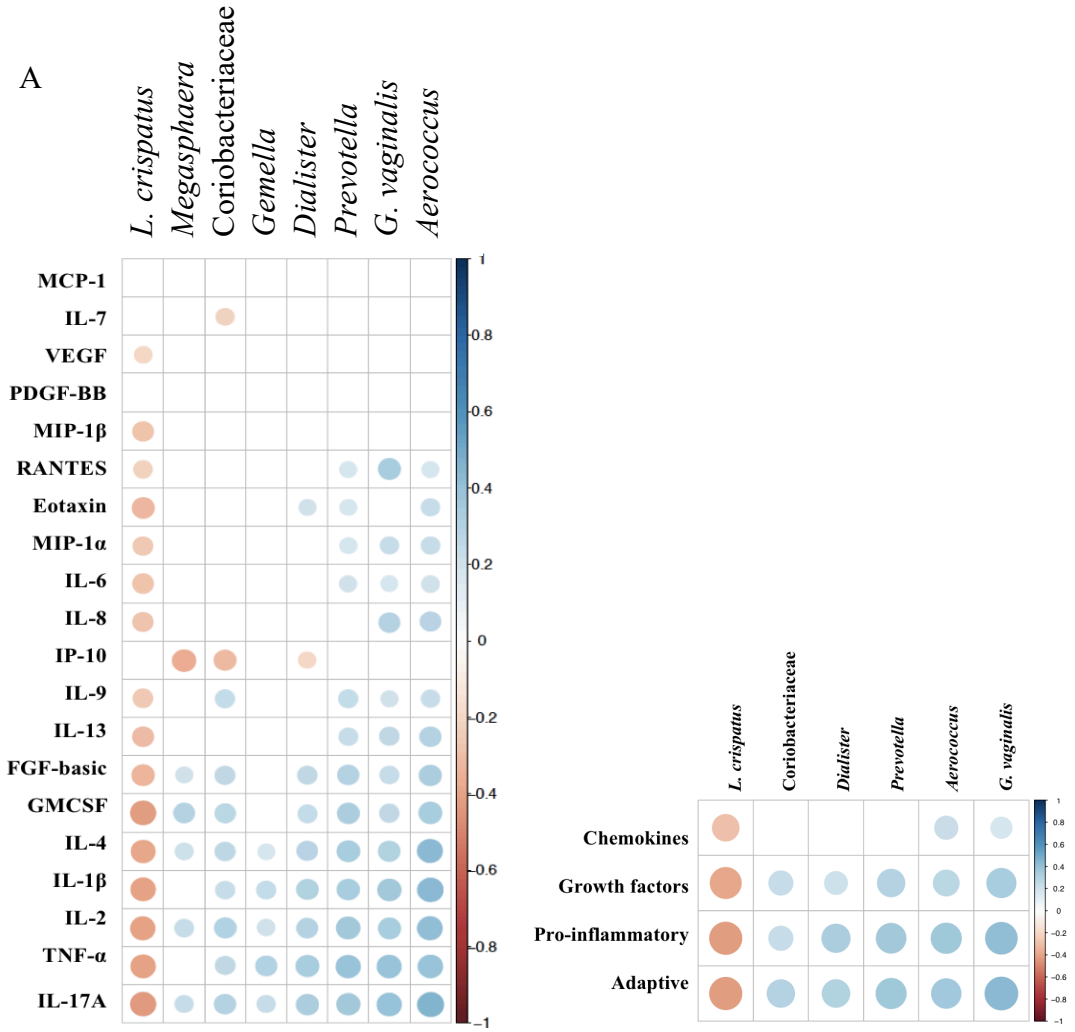


Figure 5.10 Spearman correlations between cytokines and bacterial relative abundance using taxa merged at the lowest taxonomic level. Only taxa significantly associated with cytokines shown after adjusting for multiple comparisons (adj. $p<0.05$) that had rho scores ≥ 0.3 are shown. Blue represents positive correlations while red represents negative correlations between bacterial relative abundance and vaginal concentrations of cytokines. A) Correlations between individual cytokines and 8 bacterial taxa. B) Correlations between cytokine functional groups and bacterial taxa.

5.4 Birth outcomes and immune factors

Unsupervised hierarchical clustering was used to display the relationship between vaginal cytokine concentrations and birth outcomes **(Figure 5.11).**

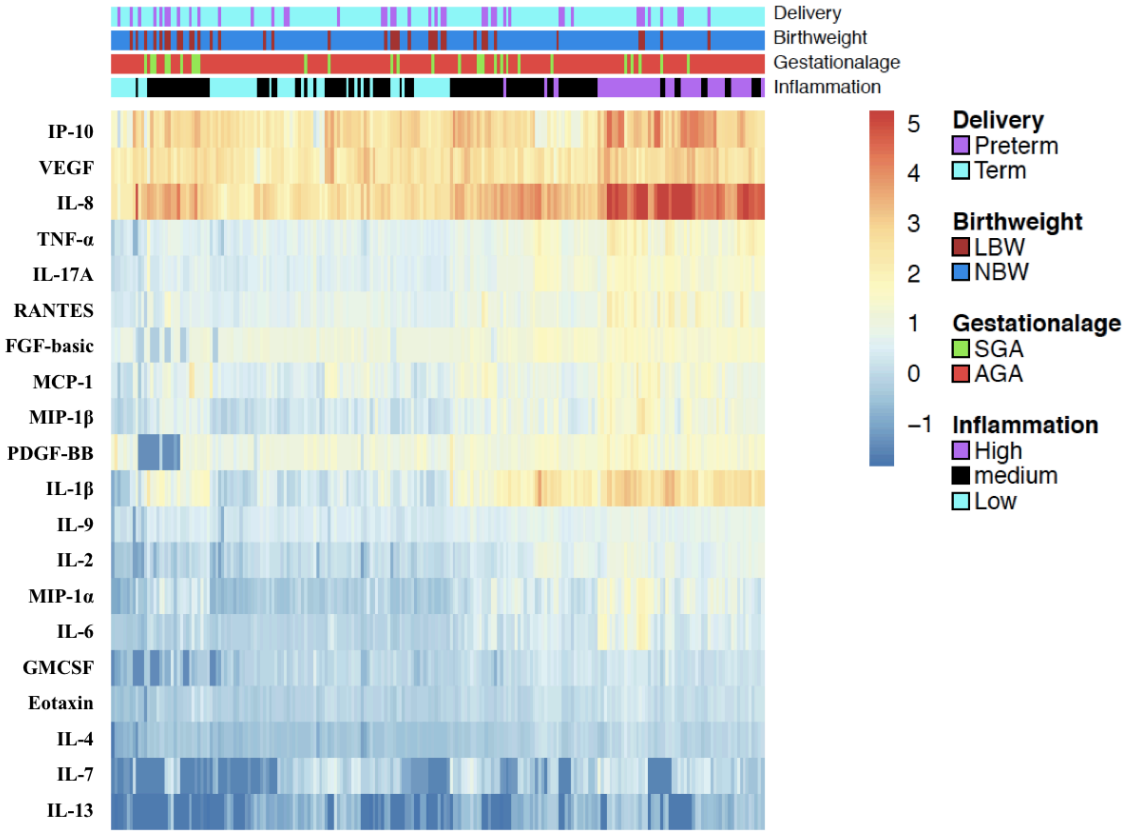


Figure 5.11 Unsupervised hierarchical clustering of all measured genital cytokines in pregnant women using $\log_{10}$ transformed cytokine concentrations. Each column represents a woman and rows represent individual cytokine. Red colour represents higher concentrations while blue presents lower concentrations. From top the figure shows clustering according to delivery (purple = preterm, cyan = term), birth weight (red = low birth weight (LBW), blue = normal birth weight (NBW), gestational age (green = small for gestational age (SGA), red = average for gestational age (AGA) and inflammation (purple = high, black = medium and cyan = low)

5.4.1 Preterm delivery and immune factors

Out of the 324 women who had cytokine analysis data, 244 women had pregnancy outcome information while of them 42 had preterm deliveries. In our univariate analysis using Mann Whitney U test (Wilcoxon rank sum test with continuity correction), growth factors and adaptive immune factors were significantly higher in women who subsequently delivered preterm, including PDGF-BB, IL-13 (both $p<0.001$) and Eotaxin, GM-CSF, FGF-basic, and IL-2 (all $p<0.005$) **(Table 5.3).**

Table 5.3 Cytokines significantly different between term and preterm delivery in univariate analysis

	Mean cytokine concentrations [log_{10}(pg/ml)]		P values	FDR adj.
	Term	Preterm		
PDGF-BB	1.261	1.041	1.56E-05	**0.0003**
IL-13	-0.769	-1.699	3.73E-05	**0.0003**
Eotaxin	-0.013	-0.041	0.0008	**0.006**
FGF-basic	1.223	1.125	0.002	**0.012**
TNF-α	0.893	0.719	0.02	0.07
IL-2	0.278	0.154	0.02	0.08
GM-CSF	0.060	-0.022	0.04	0.11

After performing a multivariate logistic regression, adjusting for possible risk factors for preterm delivery (including pregnancy-induced hypertension, trimester, maternal age, Nugent score (BV), HIV and previous poor outcomes) and log_{10} -transformed cytokine concentrations, lower levels of IL-13 (p=0.002), and PDGF-BB (p=0.041) remained significantly associated with preterm birth **(Table 5.4; Figure 5.12A).**

Table 5.4 Multivariate analysis of factors associated with preterm delivery

	OR	95% CI	Adj. p-value
IL-13	3.22	1.57-7.01	**0.002**
PDGF-BB	1.63	1.01-2.61	**0.041**
FGF-basic	2.16	0.9-5.12	0.074
Eotaxin	2.59	0.56-12.12	0.218
TNF- α	1.52	0.67-3.57	0.323
IL-2	1.31	0.55-3.07	0.531
GMCSF	1.01	0.41-2.35	0.985

Adjusted for HIV, maternal age, pregnancy induced hypertension, trimester, Nugent score (BV) and previous poor outcome

5.4.2 LBW and immune factors

Concentrations of each of the 20 cytokines were compared in vaginal secretions of women who delivered normal birth weight (NBW)(n=198) infants versus women who delivered LBW infants (n=35). Univariate analysis was performed using Mann Whitney U test (Wilcoxon rank sum test with continuity correction), a number of immune factors were associated with LBW, including PDGF-BB, FGF-basic, IL-13, IL-2, GM-CSF, TNF-α ($p<0.001$), IL-9, IL17A, Eotaxin, IL-4 and IL-7 ($p<0.05$). Overall, 9 cytokines were downregulated, while only 2 were upregulated (IL-4 and GM-CSF) in women who subsequently delivered LBW infants as compared to NBW (**Figure 5.12B)**. Growth factors and hematopoietic cytokine concentrations (PDGF-BB, FGF-basic, GM-CSF and IL-9) were significantly lower (adj. p=0.0001, p=0.001, respectively; **Table 5.5)** in women who delivered LBW babies as compared to women who delivered NBW babies.

Table 5.5 Cytokines significantly different by birth weight in univariate analysis

	Mean cytokine concentrations [log_{10}(pg/ml)]		**Pvalues**	**FDR adj. p-value**
	Normal birth weight	**Low birth weight**		
PDGF-BB	1.228	0.913	4.52E-06	**0.0001**
FGF-basic	1.226	1.061	5.50E-05	**0.001**
IL-13	-0.824	-1.699	0.000202	**0.001**
IL-2	0.335	0.061	0.0002631	**0.001**
GM-CSF	0.064	-0.142	0.00047	**0.002**
TNF-α	0.889	0.621	0.0005746	**0.002**
IL-9	0.561	0.444	0.001692	**0.005**
IL-17A	0.885	0.555	0.002859	**0.007**
Eotaxin	-0.032	-0.180	0.003726	**0.008**
IL-4	-0.301	-0.398	0.01016	**0.020**
IL-7	-0.237	-1.602	0.01358	**0.025**
IL-1β	1.522	1.015	0.05515	0.092

Multivariate logistic regression analysis was performed to evaluate the relationship between each cytokine and birth weight adjusting for possible confounders, including pregnancy-induced hypertension, maternal age, Nugent score (BV), trimester, HIV and previous poor outcomes. PDGF-BB and IL-13, remained significantly associated with LBW ($p<0.05$) **(Table 5.6: Figure 5.12B).**

Table 5.6 Multivariate logistic regression analysis of cytokines associated with low birth weight

	OR	95% CI	Adj. p-value
PDGF-BB	0.51	0.30-0.85	**0.009**
IL-13	0.36	1.42-0.88	**0.03**
FGF-basic	0.39	0.15-1.02	0.05
IL- 7	0.6	0.33-1.06	0.08
IL-2	0.51	0.19-1.39	0.19
GMCSF	0.65	2.56- 1.72	0.37
TNF-α	0.43	0.14-1.25	0.14
IL-9	0.94	0.19-4.74	0.93
Eotaxin	0.41	0.07-2.43	0.33
IL-4	0.58	1.15- 3.31	0.52
IL-17A	0.45	0.11-1.68	0.255

Adjusted for HIV, maternal age, pregnancy induced hypertension, trimester, Nugent score (BV), previous poor outcome. (CI= confidence interval, OR=odds ratio).

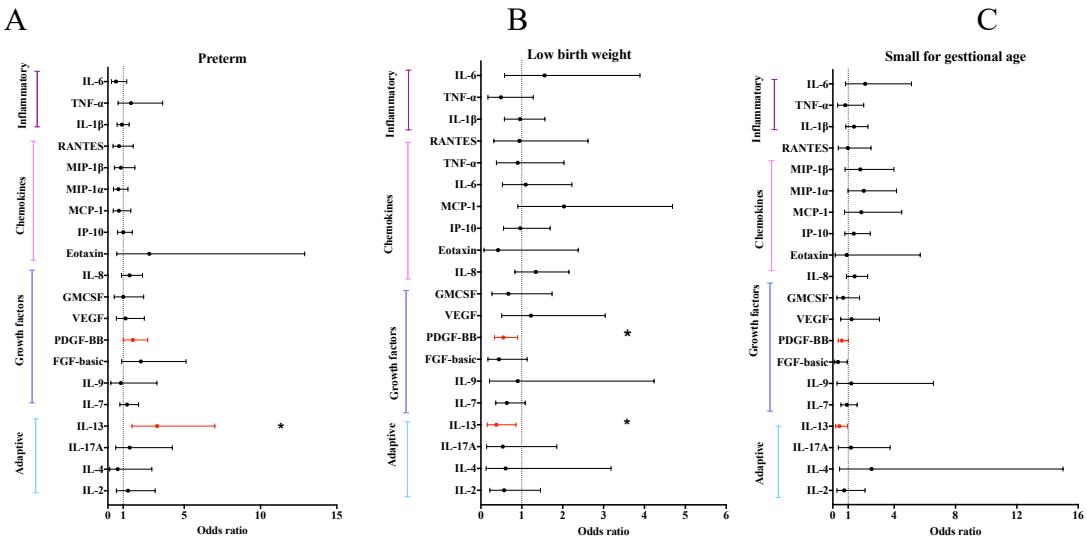


Figure 5.12 The relationship between $\log_{10}$ transformed vaginal cytokines and A. Preterm delivery, B. Low birth weight, C. Small for gestational age. Circles represent odds ratios generated using logistic regression and bars represent 95% confidence intervals. Bars and circles depicted in red represent significant associations (adj. p-values ≤ 0.05) while black represent lack of significance obtained using using logistic regression. The 20 cytokines and four functional groups of cytokines are represented on the left side. * ($p<0.05$) indicates associations that stayed significant after adjusting for possible confounders (including HIV, maternal age, pregnancy induced hypertension, trimester, Nugent score (BV) and previous poor outcome) using multivariate logistic regression.

5.4.3 Associations between cytokine concentrations and other factors

SGA was significantly associated with low levels of both IL-13 and PDGF-BB ($p<0.05$) before adjustment of multiple comparisons **(Figure 5.12 C).** However, these associations were not upheld after FDR adjustment for multiple comparisons. There were no associations observed between vaginal cytokine concentrations and GBS carriage or and still birth. When comparing cytokine functional group factor scores according to trimester, growth factors, chemokines, adaptive immune mediators and pro-inflammatory factor scores were all higher in the third trimester as compared to the second trimester **(Figure 5.13A).** Several individual cytokines were significantly upregulated in the third trimester as compared to the second trimester. FGF-basic, GM-CSF, MCP-1, MIP-1β, IL-13, IL-2 ($p<0.05$) and IL-6, RANTES and PDGF-BB ($p<0.01$). However, after FDR adjustment only PDGF-BB which remained significantly higher in third trimester as compared to second trimester $p<0.001$. There were more women in third trimester who had high (n=59; 28%) and medium (n=106; 50%) inflammation as compared to women in second trimester (22 (19%), 60 (52%) respectively). Conversely, a higher percentage of women with low inflammation were observed in the second trimester (n=33; 25%) at collection as compared to third trimester (n=46; 22%) **(Figure 5.13B).**

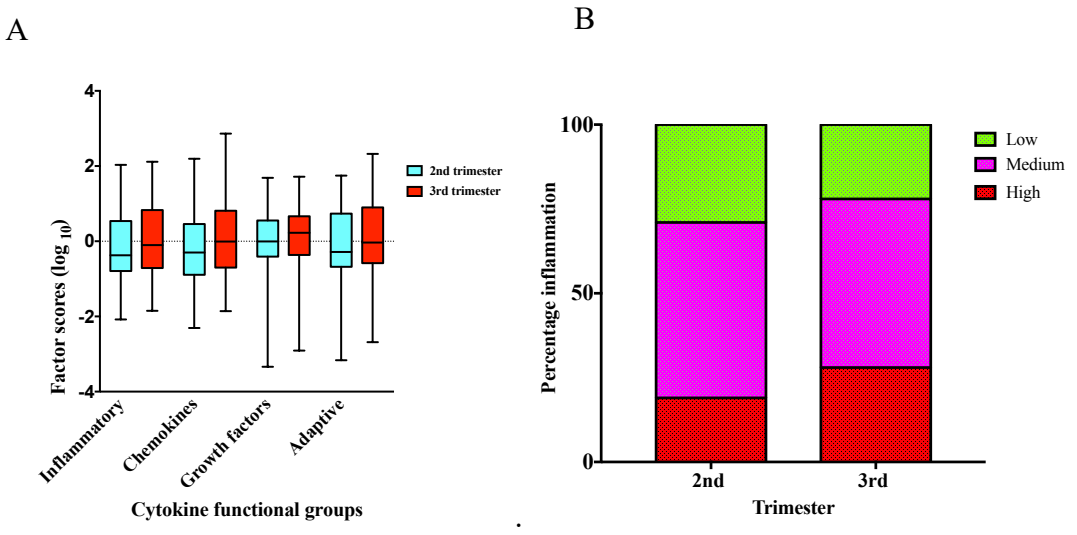


Figure 5.13 A. Cytokine functional groups by trimester. Relationships are depicted by box and whisker plots indicating the medians (middle line), 25th (bottom line) and 75th percentiles (top line), and the ranges (whiskers) of the cytokine factor score (log_{10}-transformed (pg/ml). **B.** Bar plots showing the percentages of women per trimester by inflammation categories. Confirmatory factor analysis was used to group these cytokines together and generate factor scores representing the overall level of inflammation in each woman, with women grouped as having high inflammation if their inflammatory factor score was in the upper quartile ($\geq 75^{th}$ percentile), medium inflammation if their score was in the interquartile range ($<75^{th}$ - $>25^{th}$ percentile) and low inflammation if their score was in the lower quartile ($\leq 25^{th}$ percentile.

5.4 Discussion

During pregnancy the stability and healthy development of the foetus from conception to delivery is critical. Thus, the increased incidence of adverse birth outcomes has led to a greater need to explore and identify underlying mechanisms including genital inflammation and/ or vaginal microbiota. Although causal relationships between cytokines, vaginal microbiota and pregnancy outcomes have been difficult to establish, the fact that these factors may play influential roles in pregnancy outcomes cannot be ignored. Vaginal microbiota influences vaginal cytokine profiles. Dysbiotic vaginas are highly associated with high inflammation in African women [134, 348, 412]. During pregnancy vaginal inflammation in turn is thought to be associated with poor outcomes [318, 337]. In this study, BV and high relative abundance of *L. iners* and *G. vaginalis* and other dysbiotic bacteria correlated with high levels of inflammation, whereas *L. crispatus* and other non-*iners lactobacilli* were associated with low levels of inflammation. Interestingly, lower concentrations of vaginal growth factors and hematopoietic cytokines were associated with adverse birth outcomes, including preterm delivery and LBW.

The upregulation of vaginal cytokines in women with BV that was observed in our study has been previously reported in non-pregnant adult African women [224, 237, 413]In these previous studies there was an increase in pro-inflammatory cytokines, with concomitant downregulation of chemokines. Similarly, in the present study, pro-inflammatory cytokines were upregulated and the chemokine IP-10 was downregulated in women with BV compared to women with healthy microbiota. However, several other chemokines were upregulated, as well as adaptive cytokines and growth factors in the BV+ women. Similarly, the elevation of inflammatory cytokines (IL-1β, IL-6) in BV+ normal pregnancy laboring women [414] and BV+ normal pregnancy not in labour respectively were observed in a study by Donders and colleagues [415]. Summarising our findings and previous reports, it is apparent that BV seems to be associated with high levels of proinflammatory cytokines and most chemokines in pregnancy, similar to the non-pregnant state.

We observed higher concentrations of proinflammatory cytokines (IL-1β, TNF-α, IL-8 and IL-6) in the *L. iners*-dominated and *G. vaginalis*-dominated CSTs as compared to the *L. crispatus* community. The inflammatory nature of *L. iners,* the dominant *Lactobacillus* species in this cohort, agrees with many studies that have reported that *L.*

iners, unlike other *Lactobacillus* species, may not be as "healthy" as other *lactobacilli* as it may promote a favourable environment for dysbiotic microbes [13, 67, 135]. On the other hand, *L. crispatus* was inversely associated with inflammation in this study, which agrees with findings in other African cohorts [13, 134]In other studies of non-pregnant women, it was shown that host inflammatory responses were stimulated by the presence of a dysbiotic environment, with cytokines such as IL-18, RANTES, IL-2, MIF (Macrophage migration inhibitory factor), IL-1ra, IL-8, MIP-1α and TNF-α correlating with altered vaginal composition [416]. Inflammatory cytokines may also in turn influence the vaginal microbiota, promoting the growth of opportunistic organisms[409, 411, 416].

We created an "inflammation" score that was representative of overall levels of inflammation, and binarised women into high and low inflammation categories, we observed that a high relative abundance of specific organisms such as *G. vaginalis, Dialister, A. vaginae, Aerococcus, Prevotella, Megasphaera,* and *L. iners* were found to be highly associated with inflammation. As expected, the same microbiota were highly predictive of inflammation by random forest and also significantly different in women classified as having high inflammation in a metagenomeSeq analysis. Therefore, these taxa may be possible biomarkers of inflammation during pregnancy in African women which could be validated in longitudinal studies. Of the 22 taxa significantly associated with high and low inflammation using MetagenomSeq analysis, *Prevotella Gemella, Aerococcus, A. vaginae, G. vaginalis Megasphaera, L. reuterii, L. crispatus, Dialister* and *L. iners* have been previously described as correlates of inflammation (or lack thereof) only in non-pregnant women [134, 135]. Our data adds to the literature as no study has analysd the relationship between the vaginal microbiota and inflammatory cytokines in pregnancy. We also found that some taxa that have not previously been associated with inflammation, including *P. melaninogenica, Veillonella, Parvimonas, Slackia, Roseburia* and *Nesterenkonia,* were more abundant in the high inflammation group.

When looking at specific cytokines in relation to vaginal microbiota, there were significant associations between most cytokines evaluated and individual taxa. *G. vaginalis* was highly correlated with most of the cytokines, including IL-2, IL-17A, FGF-basic, IL-9, IL-8, TNF-α, IL-1β and IL-4. This agrees with *in vitro* studies where

G. vaginalis was seen to increase IL-8 and RANTES production by female reproductive tract epithelial cells in vitro [222, 259]. Other taxa such as *Aerococcus, Prevotella* and *Coriobacteriaceae* were also strongly correlated with inflammatory and adaptive cytokines. *Prevotella* has been shown to induce secretion of inflammatory cytokines (IL-1β, IL-1α, and IL-8) *in vitro* from HeLa cells, while *Aerococcus* was associated with inflammation in the female genital tracts of non-pregnant women [135].

When looking at vaginal cytokines in the second versus third trimester, we observed upregulation of pro-inflammatory IL-6, chemokines (MCP-1, MIP-1α, MIP-1β, RANTES), growth factors (FGF-basic, GM-CSF) and adaptive immune mediators (IL-13, IL-2) during the third trimester compared to second. This concurs with reports of previous studies which found that chemokines and cytokine expression is increased in decidua tissue of pregnant women in the first and third trimesters [417]. They further reported that these increased cytokines might play a role in maternal tolerance at the maternal fetal interface [417, 418]. In contrast IL-6, IL-8 and IL-1ra were found to be downregulated in pregnant women as compared to non-pregnant although in third trimester the levels were higher compared to pre-pregnancy levels [415]. Nevertheless, sampling the same women longitudinally may better inform on the vaginal cytokine patterns during pregnancy. Maternal cytokines during pregnancy have also been investigated in several other studies with interesting observations although not necessarily they were not particularly from vaginal fluid. Upregulated serum inflammatory cytokines (IL-1β, IL-6, IL-12, IL-15, IP-10, and sCD40) have been reported to characterize the third trimester of pregnancy while an increase in serum growth factors characterises the first trimester, with a concomitant decrease in inflammatory markers IL-6, IL-17, IP-10, Eotaxin, and MCP-1 [419]. In a longitudinal study where, maternal plasma cytokines were collected from early to mid-gestation, an increase in IL-12 and IFN-γ and a decrease in IL-2 and GM-CSF levels were reported as pregnancy progressed [420]. In another longitudinal study Denney *et al., (*2011) found a consistent decrease in IFN-γ, TNF-α, IL-1β, and IL-6 throughout gestation in maternal plasma [420].

Here, we demonstrate a relationship between vaginal growth factors and poor pregnancy outcomes. We observed that LBW and PTB were significantly associated with low levels of PDGF-BB after adjusting for potential confounders. Growth factors within the maternal circulation are essential regulators of placental development and

function and have been found to be important for fetal growth [421] as previously elaborated. Previous studies have found a relationship between placental growth factors and poor pregnancy outcomes [422]. In this study we observed that lower concentrations of PDGF and to a lesser degree FGF were associated with PTB and LBW. Similarly, in other studies of placental growth factors women with low concentrations of these factors were more likely to experience poor maternal outcomes. Low placental growth factors were associated with preterm deliveries [423] while lower plasma cytokines concentrations were associated with delivery of SGA neonates [424]. However, in contrast, Smith and colleagues found that high plasma placental growth factors were associated with SGA [425] while Lee *et al.,* (2016) found that high levels of placental growth factors were associated with preterm delivery [426]. Growth factors are of paramount importance during pregnancy as they are responsible for blood cell formation and angiogenic processes that allow increase of surface area, exchange of nutrients and normal blood flow during foetal growth and development [422]. These are necessary processes for the healthy growth of the foetus. However, disruption of the normal balance of the angiogenic factors has been associated with poor pregnancy outcomes such as preeclampsia [427], IUGR, SGA [424] and preterm delivery among others. It is possible that there may be a relationship between placental and vaginal growth factors. This might explain observed associations between the growth factors PDGF-BB and PTB, LBW and SGA; and FGF-basic protein and PTB.

In prior studies [333, 410], IL-6 in cervicovaginal and amniotic fluid was strongly correlated with preterm delivery. A systematic review reported the role of subclinical infections in stimulating cytokine release during pregnancy. Proinflammatory cytokines such as IL-1α, IL-8, and IL-6 can lead to production of prostaglandins which in turn leads to premature labour [228, 410]. Furthermore, Torbe and Czajka (2004). reported high concentrations of vaginal IL-6 as the best predictive marker of preterm birth [408]. In agreement, Wei and colleagues specified that high IL-6 levels in vaginal and amniotic fluid but not in plasma was strongly associated with preterm delivery [410]. In neither IL-6 nor any other innate inflammatory cytokines were not associated with preterm delivery or any poor outcomes.

One of the functions of Th2 cytokines are as humoral immune mediators, but they also play a role in healthy pregnancies. As an adaptation during pregnancy, maternal-foetal tolerance has been suggested to be maintained by the shift of the maternal immune system from Th1 dominance to Th2. On the other hand, infection may favour cell mediated immunity (Th1 cytokines) associated with poor pregnancy outcomes [428]. As IL-13 is a Th2 and adaptive cytokine it is surprising that in our study IL-13 was highly associated with poor pregnancy outcomes (LBW, PTB and SGA) although in SGA although the association with SGA was not upheld. In contrast, as expected a recent study found that serum IL-13 and other serum cytokines were elevated in women who experienced preterm deliveries. Although they were measuring IL-13 and IL-8 in relation to prediction of preterm delivery their results could not validate that relationship [429]. These differences between studies could be attributed to the interactions between different immune factors, cytokines, diet and hormones during pregnancy. In this study it was beyond our scope to study all of the factors that influence physiological changes in pregnant women. This finding suggests that IL-13 could be a possible possible immune marker for preterm birth or poor pregnancy outcomes and should be further evaluated in future studies.

Despite multiple studies having identified biomarkers associated with PTB, as the development of PTB biomarkers for clinical practice has proved challenging due to inconsistency and lack of agreement between different studies [430, 431]. Different study design, time of sample collection, physiological and ethnic differences and presumed asymptomatic infection have been suggested to be factors underlying the inconsistent findings related to PTB predictions using biomarkers.

Our results should be interpreted with caution considering our cross-sectional study design. A longitudinal study with follow up throughout pregnancy would provide a better predictive model of factors associated with adverse birth outcomes. The lack of precise gestational age determination at collection due to the inability of many women to access ultrasound services could also affected the results and influenced trimester and gestational age classification. Another drawback is that half of the women in this cohort had high risk pregnancies since they had experienced multiple previous poor pregnancy outcomes. Although this did increase the proportion of women with adverse outcomes, the data may lack generalisability. Although all women were tested for STIs

(syphilis, T. vaginalis and HIV) during the visit, we were unable to control for other STIs.

In conclusion, we find that vaginal cytokine concentrations vary during pregnancy depending on gestational age and vaginal microbiota state and may in turn influence pregnancy outcomes. Our study has established that dysbiotic vaginal microbiota and BV are associated with elevated cytokine concentrations and high inflammation, although high inflammation was not associated with any poor pregnancy outcomes. Furthermore, low growth factors and Th2 cytokines were highly associated with poor birth outcomes.

Chapter 6: HIV and vaginal microbiota during pregnancy

HIV infected women have a high prevalence of vaginal dysbiosis when compared to HIV uninfected women [27]. According to published studies, the prevalence of an altered vaginal microbial environment in HIV-infected pregnant women is between 47% in western countries and 89% in some African cohorts [14]. HIV infection is associated with increased risk of poor pregnancy outcomes in pregnant women on ART[56, 178, 180, 432]. An important underlying mechanism for the relationship between HIV and poor pregnancy outcomes may be changes in the vaginal microbiota and/or inflammatory profile as a result of HIV infection [143, 433]. Previous studies in non-pregnant women have shown that HIV causes alterations in the vaginal microbiome and increases in pro-inflammatory cytokine concentrations [221, 224, 348]. HIV-1 is known as an inflammatory condition that is associated with dysregulated and imbalanced cytokine production at mucosal and systemic sites which is a hallmark of disease progression [164, 434]. During pregnancy, placental concentrations of inflammatory cytokines, including TNF-α, IL-6 and IL-8, have been found to be elevated in HIV-infected women on ART as compared to HIV-uninfected women [435, 436] as fully elaborated in Chapter 1. The use of HAART on cytokine levels in serum of HIV-infected pregnant women and found that HAART has a suppressive effect on circulating pro-inflammatory cytokines levels [437-439].

The vaginal microbiota have also been associated with increased risk of HIV transmission [143]Additionally, studies conducted by Roberts *et al., (*2012) and Cortez *et al.,* (2014) in non-pregnant HIV-infected women both demonstrated that vaginal inflammatory cytokines (TNF-α, IL-6, MIP-1α, IFN-α2 and MIP-1β) were associated with increased viral shedding [164] {Cortez, 2014 #728. Therefore, changes in the vaginal microbiome and inflammatory markers during pregnancy may influence the likelihood of mother-to-child transmission of HIV and transmission to their uninfected partners.

In order to improve our understanding of the changes in vaginal microbiota and cytokines that occur during HIV infection that may influence pregnancy outcomes and HIV transmission, in this Chapter we compare the vaginal microbiota and cytokine profiles of HIV infected and HIV uninfected pregnant women between 13-35 weeks of gestation.

Out of the 420 women recruited, 356 women had complete data on HIV status and microbiota. Of these 356 women, 314 (88%) were HIV-uninfected and 42 (12%) were HIV-infected **(Table 6.1).** There were no differences in characteristics such as gestational age at collection, parity, GBS colonization, vaginal douching, vaginal pH and gravida between the two groups **(Table 6.1).** Of the 42 HIV-infected women, 24 had available CD4+ counts and 8 had viral load results, of which the mean CD4+ count was 532.8 cells/mm^3 and viral load was 128.85 copies/ml. The majority of these women were on the fixed dose combination drug which consist of Tenofovir (300 mg) + Lamivudine (300 mg) + Efavirenz (600 mg) (Tenolam E) [35/42 (87,5%)] HAART regimen. Preterm deliveries tended to be more frequent in HIV-infected women compared to HIV-uninfected women (31% vs 15.3%, p=0.066 respectively). Women who were HIV-infected were more likely to have a smoking partner (p=0.03) and have BV by Nugent score (p=0.01) compared to HIV-uninfected women.

Table 6.1 Characteristics stratified by HIV Status

	HIV Negative (n=314)	**HIV Positive (n=42)**	**p-value**
Gestational Age collection, mean (SD)	28.32 (6.44)	28.64 (5.00)	0.754
Partner smoker (%)	19 (6.1)	7 (16.7)	**0.030**
SGA (%)	25 (12.2)	5 (17.9)	0.590
Blood CD4+ [cell/mm^3(n=24)] [mean (SD)]	NA	532.8 (208.2)	
Plasma viral load copies/ml (n=8), mean (SD)	NA	128.85 (98.49)	
HAART Regimen (%)			NA
AZT 3TC	NA	4 (10.0)	
Tenofovir	NA	1 (2.5)	
TDF, FTC, EFV	NA	35 (87.5)	
LBW (%)	28 (13.7)	7 (25.0)	0.196
Preterm (%)	33 (15.3)	9 (31.0)	0.066
Vaginal douching [n (%)]	148 (47.1)	26 (61.9)	0.102
Nugent score, mean (SD)	2.98 (3.43)	4.36 (3.63)	**0.016**
BV (%)			**0.010**
BV	70 (22.3)	18 (42.9)	
Intermediate	50 (15.9)	7 (16.7)	
Normal	194 (61.8)	17 (40.5)	
pH, mean (SD)	4.18 (0.67)	4.36 (0.76)	0.124
Gravida, mean (SD)	2.82 (1.69)	3.26 (1.33)	0.102
Para, mean (SD)	1.26 (1.20)	1.67 (1.20)	**0.041**
Maternal age, mean (SD)	29.02 (6.39)	31.05 (6.41)	0.066
CST (%)			**0.009**
1	95 (30.3)	14 (33.3)	
2	98 (31.2)	4 (9.5)	
3	121 (38.5)	24 (57.1)	
Pregnancy induced hypertension (%)	21 (6.7)	0 (0.0)	0.168
Previous poor outcome (%)	97 (30.9)	12 (28.6)	0.898
GBS (%)	123 (39.2)	16 (38.1)	1.000
Inflammation (%)			0.447
High	70 (24.8)	11 (26.2)	
Low	72 (25.5)	7 (16.7)	
Medium	140 (49.6)	24 (57.1)	

LBW= low birth weight, GBS= group B streptococcus. BV=bacterial vaginosis, CST= community state type.

SGA= small for gestational age. SD= Standard deviation, NA= not applicable

A multivariate model was used to further evaluate correlates of HIV infection, including those identified in prior literature, including maternal age {Liuzzi, 2013 #750}, vaginal douching [440], and those that were significant in a univariate model **(Table 6.1).** BV status and CST were excluded due to their collinearity with Nugent score. Nugent score [OR 1.14; 95% CI 1.03-1.26] (p=0.01) and having a partner who smoked [OR 3.57; 95% CI 1.25-9.39] (p=0.01) were significantly independently associated with HIV status **(Table 6.2)**.

Table 6. 2 Multivariate analysis of demographic, behavioural and clinical factors associated with HIV status

	OR	95% CI	p-value
Nugent score	1.14	1.03-1.26	**0.01**
Para	1.18	0.9-1.61	0.30
Partner smoker	3.57	1.25-9.39	**0.01**
Vaginal douching	1.74	0.85-3.64	0.13
Maternal age	1.05	0.98-1.12	0.13

CI= confidence interval, OR= Odds ratio

Correlates of HIV infection were further evaluated, including CST and those identified in prior literature, including maternal age [441], vaginal douching [440], and those that were significant in a univariate model **(Table 6.1).** Nugent score were excluded due to their collinearity with CST. CST3 [OR 4.90; 95% CI 1.64-14.60 (p=0.002), CST1 [OR 3.57; 95% CI 1.14-11.24 (p=0.02) and having a partner who smoked (p<0.03) [OR 3.57; 95% CI 1.25-9.39] were significantly independently associated with HIV status **(Table 6.3)**. With CST2 set as the reference, CST3 and CST1 and smoking partner were independently associated with HIV (p<0.05) **(Table 5.2).** Women with CST3 microbiota were 4.90 times more likely to be HIV positive compared to women in CST2

Table 6. 3 Multivariate analysis of demographic, behavioural and clinical factors associated with HIV status

	OR	95% CI	p-value
CST3	4.90	(1.64, 14.60)	**0.002**
CST1	3.57	(1.14, 11.24	**0.02**
Para	1.20	0.88-1.66	0.21

Partner smoker	3.89	1.35-10.41	**0.01**
Vaginal douching	1.76	0.85-3.74	0.13
Maternal age	1.04	0.97-1.10	0.27

CST= community state type

Using hierarchical clustering of taxa merged at the lowest taxonomic level, the women in this cohort clustered into three CSTs as described previously in Chapter 3: CST1 (*L. iners* dominant), CST2 (*L. crispatus* dominant) and CST3 (*G. vaginalis* dominant*)*. HIV-infected women were less likely to have CST2 microbiota and more likely to have CST3 microbiota (p=0.009) than HIV negative women **(Table 6.1)**. A CST distribution analysis revealed that the majority of HIV-infected women were found in with CST3 [24/42 (57%)], followed by CST1 [12/42 (33%)], confirming microbial differences by HIV infection status. Moreover, CST2 was rarely observed in HIV-infected women [4/42 (9.5%)] as compared to HIV uninfected women [98/314 (31.2%)] **(Figure 6.1; Table 6.1)**.

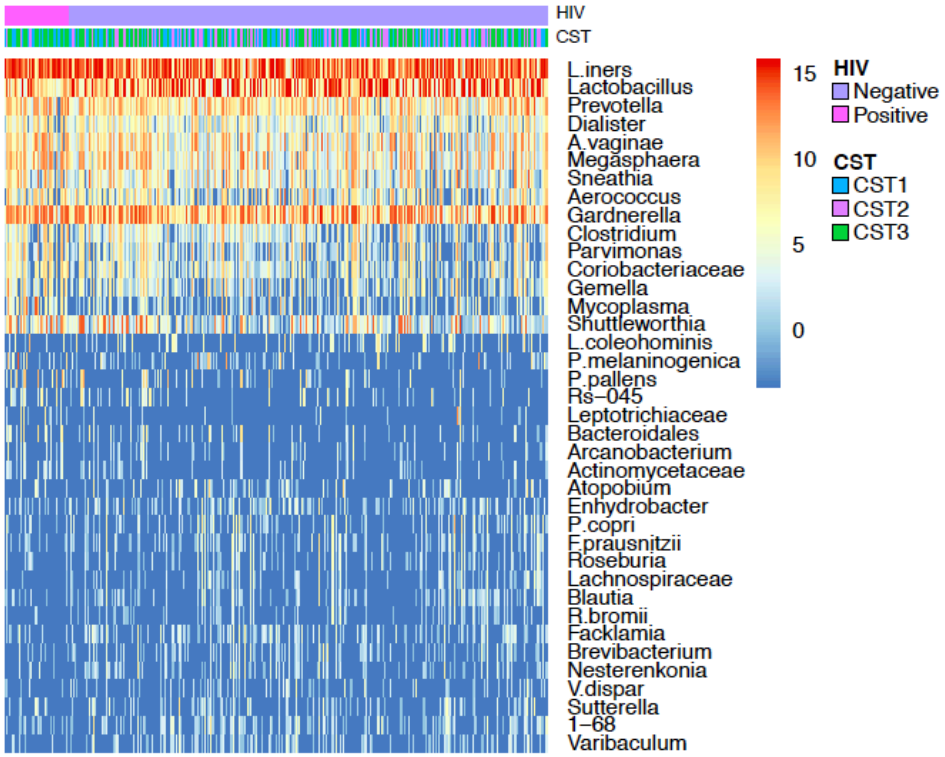


Figure 6. 1 Heatmap showing community state type (CST) distribution by HIV status using supervised hierarchical clustering (Bray Curtis distances) of $\log_2$ transformed standardized counts of taxa merged at the lowest taxonomic level. Each column represents a woman and rows represent taxa or operational taxonomic units (OTU). Red colour represents the most abundant while blue presents least abundant or absence of $\log_2$ transformed OTU counts.

Significant differences in microbial diversity (alpha and beta-diversity) were evident between HIV-infected and HIV-uninfected women (Shannon index p=0.004 and Bray

Curtis Adonis p=0.003, respectively) **(Figure 6.2A & B).** A low relative abundance of the *Lactobacillus* genus with high relative abundance of some BV-associated organisms was observed in HIV-infected women **(Figure 6.2C)**. These findings were consistent with the findings of diversity measures we previously described where HIV infection was more closely associated with CST3 than the other CSTs.

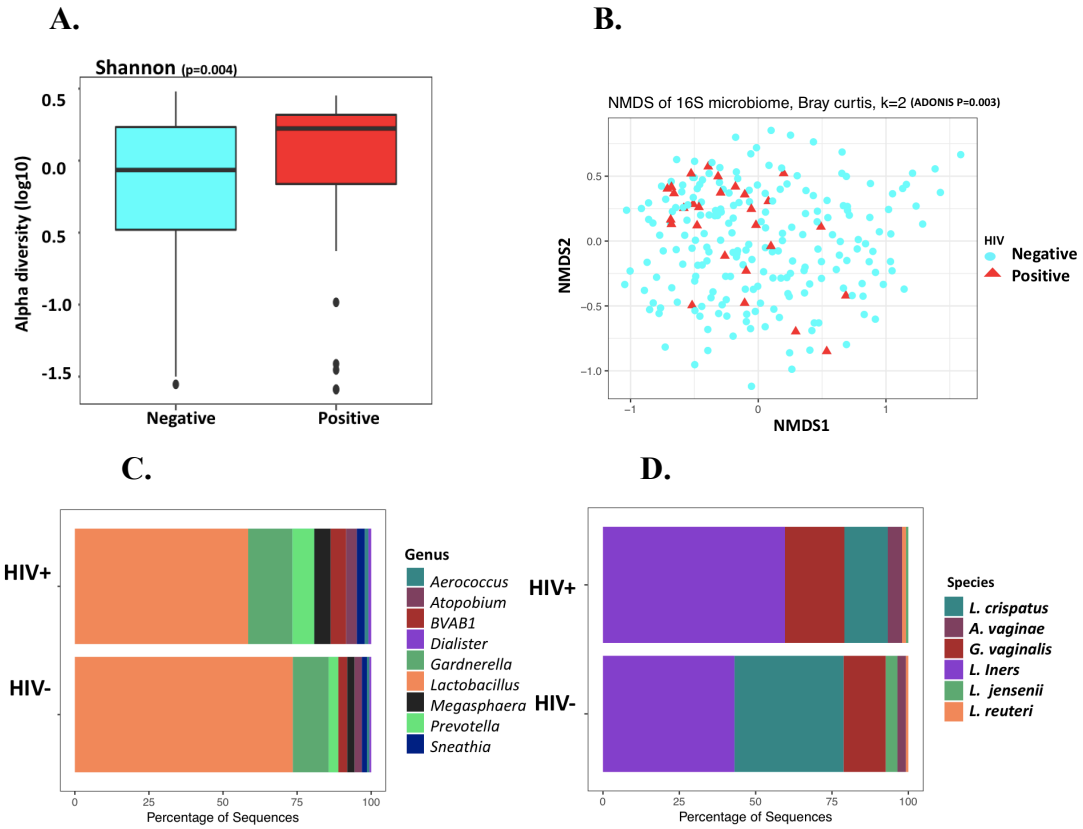


Figure 6. 2 Diversity and composition of the vaginal microbiota by HIV status. **A**. Shannon alpha diversity of the vaginal microbiota in HIV-infected and HIV-uninfected women **B.** Bray Curtis beta diversity in HIV infected and uninfected women. Results were significant by Kruskal-wallis and evaluated by Dunn's post hoc test. **C & D**. Bar plots showing relative abundance of the most abundant bacteria by HIV status at genus and species levels, respectively

Thirty-eight OTUs were identified as being significantly different between HIV-infected vs. HIV-uninfected women using MetagenomSeq **(Table 6.4 and Table 6.5).** At species level there was a higher relative abundance of *L. iners* in HIV-infected women, while conversely *L. crispatus* was higher in HIV-uninfected women as compared to the other group **(Table 64).**

Table 6. 4 Differentially abundant taxa by HIV status (Family, genus and species level where available)

Family	Genus	Species	HIV-uninfected (N=314) % (n)	HIV-Infected (N=42) % (n)	Adj. p-value
Lactobacillaceae	*Lactobacillus*	*coleohominis*	21.3 (67)	14.3 (6)	**<0.001**
Lactobacillaceae	*Lactobacillus*	*crispatus*	100 (314)	100 (42)	**0.013**
Lachnospiraceae	*NA*	*NA*	26.8 (84)	7.1 (3)	**<0.001**
Actinomycetaceae	*Varibaculum*	*NA*	33.8 (106)	11.9 (5)	**<0.001**
Lachnospiraceae	*Blautia*	*NA*	32.5 (102)	19 (8)	**<0.001**
Prevotellaceae	*Prevotella*	*copri*	28.7 (90)	21.4 (9)	**<0.001**
Ruminococcaceae	*Ruminococcus*	*bromii*	23.2 (73)	4.8 (2)	**<0.001**
Aerococcaceae	*Facklamia*	*NA*	37.3 (117)	21.4 (9)	**<0.001**
Veillonellaceae	*Veillonella*	*dispar*	20.7 (65)	11.9 (5)	**<0.001**
[Tissierellaceae]	*Jan-68*	*NA*	35.7 (112)	33.3 (14)	**<0.001**
Brevibacteriaceae	*Brevibacterium*	*NA*	30.3 (95)	16.7 (7)	**<0.001**
Ruminococcaceae	*Faecalibacterium*	*prausnitzii*	31.8 (100)	26.2 (11)	**<0.001**
Coriobacteriaceae	*Atopobium*	*NA*	21.7 (68)	11.9 (5)	**<0.001**
Lachnospiraceae	*Roseburia*	*NA*	25.8 (81)	16.7 (7)	**<0.001**
Micrococcaceae	*Nesterenkonia*	*NA*	28.3 (89)	11.9 (5)	**<0.001**
Moraxellaceae	*Enhydrobacter*	*NA*	37.6 (118)	28.6 (12)	**<0.001**
Alcaligenaceae	*Sutterella*	*NA*	25.5 (80)	16.7 (7)	**<0.001**
Lactobacillaceae	*Lactobacillus*	*iners*	100 (314)	100 (42)	**0.023**
Veillonellaceae	*Dialister*	*NA*	94.3 (296)	88.1 (37)	**0.009**
Bifidobacteriaceae	*Gardnerella*	*NA*	100 (314)	100 (42)	**0.025**
Prevotellaceae	*Prevotella*	*timonensis*	99.4 (312)	97.6 (41)	**0.012**
Aerococcaceae	*Aerococcus*	*NA*	82.2 (258)	85.7 (36)	**0.009**
Actinomycetaceae	*NA*	*NA*	13.4 (42)	40.5 (17)	**<0.001**
Leptotrichiaceae	*Sneathia*	*NA*	93 (292)	90.5 (38)	**0.006**
Clostridiaceae	*Clostridium*	*NA*	78.7 (247)	81 (34)	**0.001**
Coriobacteriaceae	*Atopobium*	*vaginae*	97.1 (305)	97.6 (41)	**0.005**
Prevotellaceae	*Prevotella*	*melaninogenica*	22.9 (72)	31 (13)	**<0.001**
Lachnospiraceae	*BVAB-1*	*NA*	87.6 (275)	95.2 (40)	**0.003**
Rs-045	*NA*	*NA*	14.6 (46)	31 (13)	**<0.001**
Gemellaceae	*Gemella*	*NA*	66.9 (210)	71.4 (30)	**<0.001**
Coriobacteriaceae	*NA*	*NA*	76.8 (241)	83.3 (35)	**<0.001**
[Tissierellaceae]	*Parvimonas*	*NA*	70.7 (222)	78.6 (33)	**<0.001**
Actinomycetaceae	*Arcanobacterium*	*NA*	15.6 (49)	23.8 (10)	**<0.001**
Leptotrichiaceae	*NA*	*NA*	5.7 (18)	23.8 (10)	**<0.001**
Veillonellaceae	*Megasphaera*	*NA*	97.5 (306)	97.6 (41)	**<0.001**

Mycoplasmataceae	*Mycoplasma*	*NA*	48.4 (152)	71.4 (30)	**<0.001**
Prevotellaceae	*Prevotella*	*pallens*	15.9 (50)	33.3 (14)	**<0.001**

L. iners, G. vaginalis, Megasphaera, P. timonensis, BVAB1*, A. vaginae, Sneathia, Mycoplasma, P. melaninogenica, Lachnospiraceae*, *Actinomycetaceae*, Coriobacteriaceae*, Gemella, P. pallens, Aerococcus,* Rs-045, *Parvimonas, Clostridium, Dialister, Bacteroidales, Arcanobacterium* and *L. coleohominis* were more abundant in HIV-infected women than HIV-uninfected (adj. p<0.05) **(Figure 6.3).** *P. copri,* Leptotrichiaceae, *Atopobium*, *Facklamia, Roseburia, R. bromii, Brevibacterium, F. prausnitzii*, Enhydrobacter, *Varibaculum, Blautia*, *V. dispar*, Jan-68, *Nesterenkonia* and, *Sutterella* were less abundant in HIV-infected compared to HIV-uninfected women (adj. p<0.05).

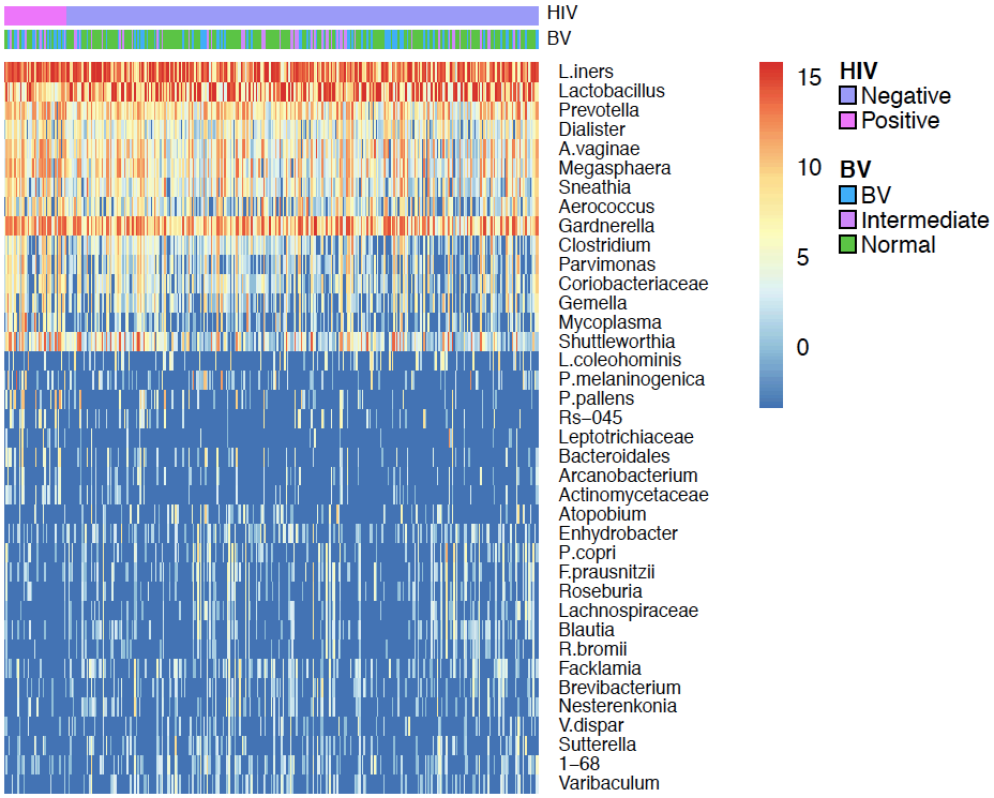


Figure 6.3 Supervised hierarchical clustering (Bray Curtis distances) including significant differentially abundant taxa by HIV status identified using MetagenomSeq analysis. Taxa were merged at the lowest taxonomic level and $\log_2$-transformed standardized read counts are shown. HIV and bacterial vaginosis (BV) status are shown. Each column represents a woman and rows represent taxa or operational taxonomic units (OTU). Red colour represents the most abundant while blue presents least abundant or absence of $\log_2$ transformed OTU counts.

We compared the standardized read counts of individual taxa predicted by RF in HIV-infected versus uninfected women to see the direction of the relationship Using Mann Whitney U test for independent samples to compare differences between the means by HIV status. It is interesting to note that *Mycoplasma* (p=0.0002), *Megasphaera* (p=0.0004) Coriobacteriaceae (p=0.001), *Parvimonas* (p=0.001), *A. vaginae* (p=0.01), *Dialister* (p=0.02), *P. timonensis* (p=0.02), *Clostridium* (p=0.03), Actinomycetaceae (p=0.03), *Corynebacterium* (p=0.03), *Aerococcus* (p=0.03) and *Prevotella* (p=0.04) read counts were significantly higher in HIV-infected compared to uninfected women, while *L. crisptatus* (P=0.03) was significantly higher in HIV-uninfected women **(Figure 6.4).** *L. iners*, *G. vaginalis, Peptoniphilus*, *P. pallens* and *P. melaninogenica*, ($p>0.05$) had non-significantly higher read counts in HIV-infected women, while and *Anaerococcus* ($p>0.05$) had a non-significantly higher read count in HIV-uninfected compared to infected woman. Only *Mycoplasma* and *Megasphaera* remained significant ($p<0.05$) after adjusting for multiple comparisons.

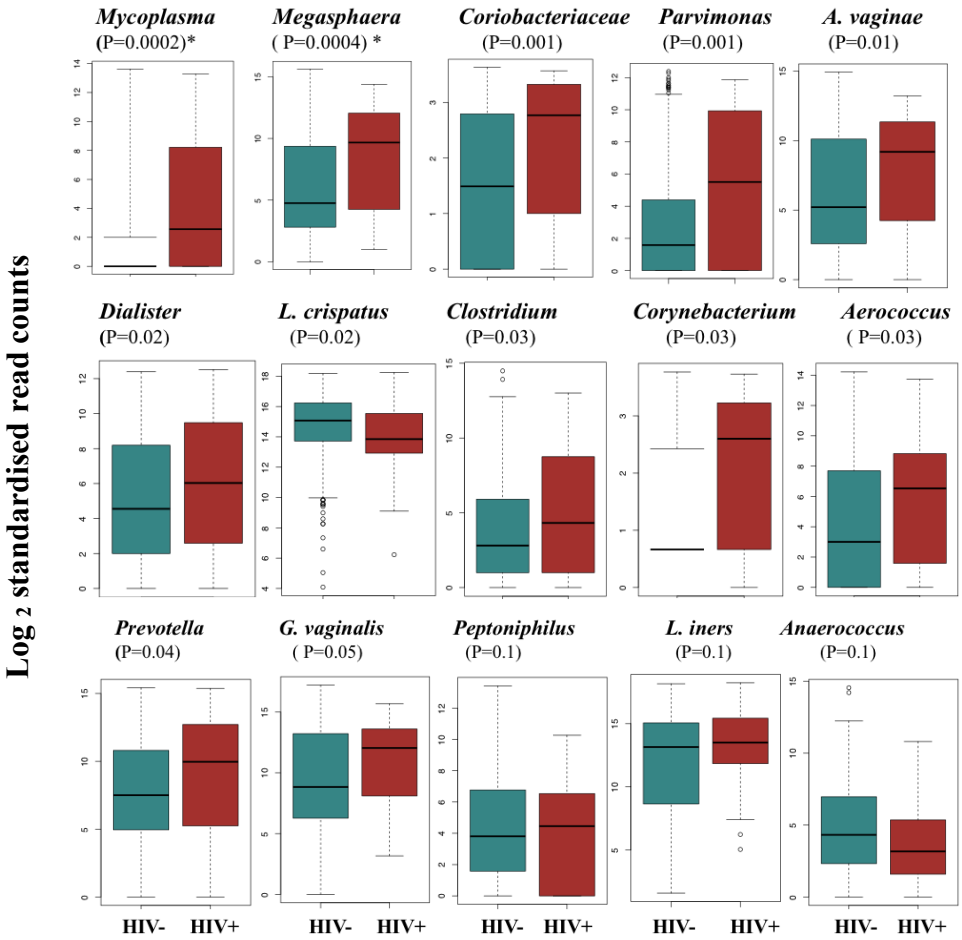


Figure 6.4 Box plot summaries log_2-transformed standardised read counts of individual taxa that differed according to HIV status in random forest. Box and whisker plots indicate the median (middle line), 25th (bottom line) and 75th percentiles (top line), and the range (whiskers) of log_2-transformed taxa read counts. Mann Whitney U test for independent samples was used for comparisons of read counts by HIV status. All p-values were adjusted using the false discovery rate (FDR) step-down approach (Columb and Sagadai, 2006). * shows p-values which were significant after multiple comparisons correction. *Corynebacterium* and *Mycoplasma* had interquartile ranges of zero thus the graphs are not displaying for the HIV uninfected women

MetagenomeSeq results were supported by Random forest (RF) analysis **(Figure 6.5).** The ten genera found to be most predictive of HIV status by RF in order were *Prevotella, Actinomycetaceae, L. iners, G. vaginalis, Dialister, Coriobacteriacae, A. vaginae, Megasphaera, Peptoniphillus, Aerococcus*. The model resulted in a 10-fold cross validation error of 12.8% (87% accuracy, 3% sensitivity and 99% specificity). However, the positive predictive value was only 33%, indicating that the model missed 77% of the HIV positive samples. *Prevotella* was the most predictive of HIV status in this cohort with a mean decrease Gini index >0.4 **(Figure 6.5).**

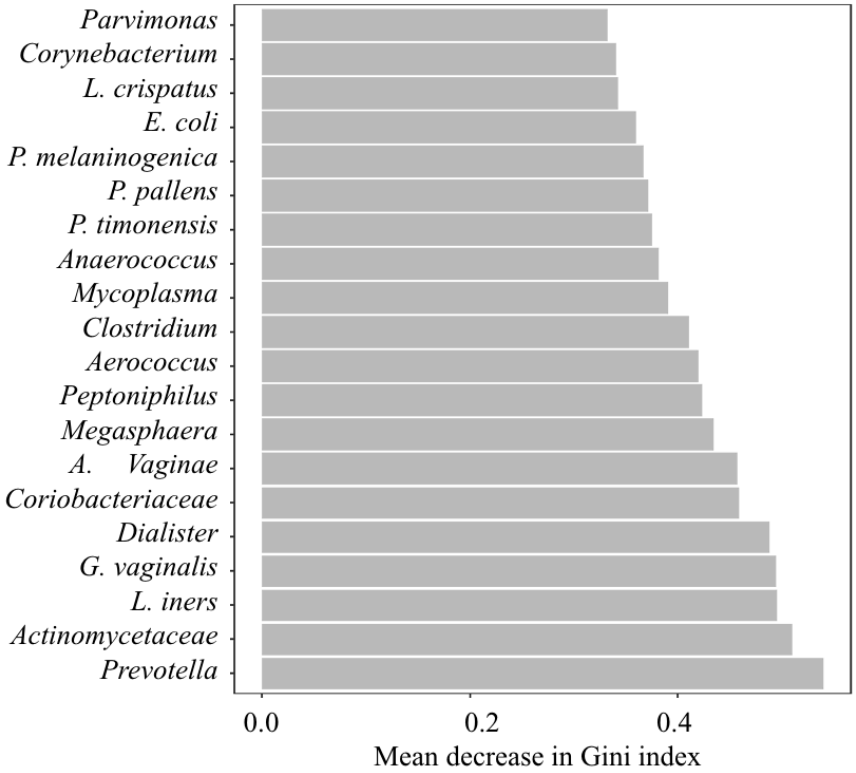


Figure 6.5 Random forest using taxa merged at the lowest taxonomic level showing the taxa predictive of HIV status. The X-axis shows the mean decrease in the Gini index (length of bar represents relative predictive ability of each taxon). The left shows taxa predictive of HIV status

6.1 HIV and vaginal cytokines

We sought to explore vaginal cytokine levels and their relationship with microbiota and HIV status in pregnant women. As described in the previous Chapter 5, the concentrations of 20 cytokines, representing four functional groups, adaptive (IL-2, IL-13, IL-4, IL-17A), regulatory (IL-10, IL-1ra), growth factors (IL-7, IL-9, FGF-basic, PDGF-BB, VEGF, GM-CSF), chemokines (IL-8, Eotaxin, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES) and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), were measured in vaginal secretions collected from 356 women. Of these cytokines, IL-4, IL-17 and IL-13 were found at very low concentrations in both HIV-uninfected and infected women, while the remainder was readily detectable. A larger proportion of HIV-uninfected women were grouped as having low levels of genital inflammation as defined in Chapter 5 as compared to HIV-infected women (16 vs 25%, respectively), but this was not statistically significant (p=0.45) **(Table 6.1)**. In a univariate analysis, only PDGF-BB was significantly lower in HIV-infected compared to uninfected women, however this association was not upheld after adjusting for multiple comparisons (p=0.02, adjusted p=0.2) **(Table 6.5).**

We went on to perform multivariate logistic regression in order to adjust for potential confounders that may influence genital cytokine concentrations and/or HIV status [440, 441] as well as factors that were associated with HIV in the univariate analysis in Table 6.1. Importantly, since microbiota and genital cytokines are so closely linked, we included variables to account for this including Nugent score in Model 1 and CST in Model 2. It was found that the relationship between PDGF-BB and HIV status was not upheld after adjusting for potential confounders including Nugent score, partner smoker, parity (p>0.05), while low levels of IL-13 (p=0.01) [OR 0.42; CI (0.21-0.82)] were found independently associated with HIV status after adjustment **(Table 6.5).**

Table 6.5 Multivariate analysis of cytokines associated with HIV status

	OR	95% CI	p-value	Adj. OR	Adj. 95% CI	Adj. p-value
IL-2	2.77	0.20-57.80	0.48	1.10	0.48-2.69	0.82
IL-4	0.13	0002-8.13	0.34	1.23	0.28-6.2	0.79
IL-17A	1.13	0.07-18.09	0.93	1.27	0.51-3.07	0.60
IL-13	0.24	0.09-0.57	0.08	0.43	0.21-0.82	**0.01***
IL-7	0.90	0.50-1.62	0.73	0.75	0.38-1.05	0.08
IL-9	0.35	0.02-6.59	0.46	0.60	0.17-2.55	0.46
FGF-basic	0.56	0.13-2.90	0.46	0.72	0.3-1.92	0.47
PDGF-BB	0.99	0.47-2.17	**0.02**	0.72	0.47-1.14	0.2
VEGF	0.45	0.17-1.14	0.09	0.86	0.44-1.66	0.64
GM-CSF	3.99	0.70-24	0.12	0.97	0.4-2.73	0.95
IL-8	1.58	0.71-3.51	0.26	1.25	0.86-1.8	0.23
Eotaxin	1.41	0.08-26.81	0.82	0.74	0.19-3.19	0.67
IP-10	0.80	0.41-1.54	0.50	1.14	0.75-1.74	0.53
MCP-1	0.32	0.06-1.54	0.18	0.84	0.36-1.86	0.67
MIP-1α	56.7	5.92-628.09	0.19	1.25	0.68-2.28	0.47
MIP-1β	0.08	0.01-0.73	0.70	1.05	0.53-2.05	0.87
RANTES	3.72	0.83-16.69	0.08	1.68	0.77-3.53	0.18
IL-1β	0.45	0.19-1.18	0.07	1.18	0.8-1.73	0.40
IL-6	0.12	0.02-0.6	0.72	0.64	0.27-1.38	0.28
TNF-α	4.13	0.80-21.46	0.09	1.38	0.66-2.87	0.38

* significant after adjusting for Nugent score, partner smoker status, maternal age, para, vaginal douching. Cytokine concentrations were log_{10}-transformed.

Additional multivariate logistic regression analyses were performed in order to adjust for CST instead of Nugent score, as CST and Nugent score could not be included in the same model due to collinearity. Similarly, the relationship between PDGF-BB (p=0.38), and HIV status were not upheld after adjusting for potential confounders including CST, maternal age, partner smoker, parity while high levels of TNF- α (p=0.04) [OR 2.48; CI (1.06-5.89)] were associated with HIV. **(Table 6.6)**

Table 6.6 Multivariate analysis of cytokines associated with HIV status

	OR	Adj. OR	95% CI	Adj. 95% CI	p-value	Adj. p-value
IL-2	2.77	2.59	0.20-57.80	0.97-7.69	0.48	0.07
IL-4	0.13	3.13	0002-8.13	0.57-20.11	0.34	0.21
IL-17A	1.13	2.37	0.07-18.09	0.89-6.51	0.93	0.09
IL-13	0.24	0.7	0.09-0.57	0.32-1.47	0.08	0.34
IL-7	0.90	0.84	0.50-1.62	0.48-1.46	0.73	**0.29**
IL-9	0.35	1.76	0.02-6.59	0.33-12.84	0.46	0.54
FGF-basic	0.56	4.44	0.13-2.90	0.1-25.99	0.46	0.07
PDGF-BB	0.99	0.79	0.47-2.17	0.46-1.42	0.02	0.38
VEGF	0.45	0.48	0.17-1.14	0.2-1.09	0.09	0.09
GM-CSF	3.99	3.08	0.70-24	0.87-15.48	0.12	0.12
IL-8	1.58	1.4	0.71-3.51	0.89-2.18	0.26	0.14
Eotaxin	1.41	1.01	0.08-26.81	0.22-5.51	0.82	0.98
IP-10	0.80	0.94	0.41-1.54	0.55-1.6	0.50	0.81
MCP-1	0.32	0.7	0.06-1.54	0.23-1.97	0.18	0.51
MIP-1α	56.7	1.38	5.92-628.09	0.67-2.84	0.20	0.38
MIP-1β	0.08	1.26	0.01-0.73	0.57-2.76	0.70	0.57
RANTES	3.72	2.22	0.83-16.69	0.88-5.4	0.08	0.08
IL-1β	0.45	1.39	0.19-1.18	0.91-2.17	0.07	0.13
IL-6	0.12	1.14	0.02-0.6	0.46-2.71	0.90	0.77
TNF-α	4.13	2.48	0.80-21.46	1.06-5.89	0.09	**0.04***

*significant after adjusting for community state type (CST), partner smoker status, maternal age, para, vaginal douching

A heatmap was constructed to visualize the relationship between the vaginal microbiota and cytokines (**Figure 6.6).** Included in the heatmap were inflammatory scores assigned by grouping women as having high inflammation if their inflammatory factor score was in the upper quartile (≥ 75th percentile), medium inflammation if their score was in the interquartile range (<75th - >25th percentile) and low inflammation if their score was in the lower quartile (≤25th percentile).

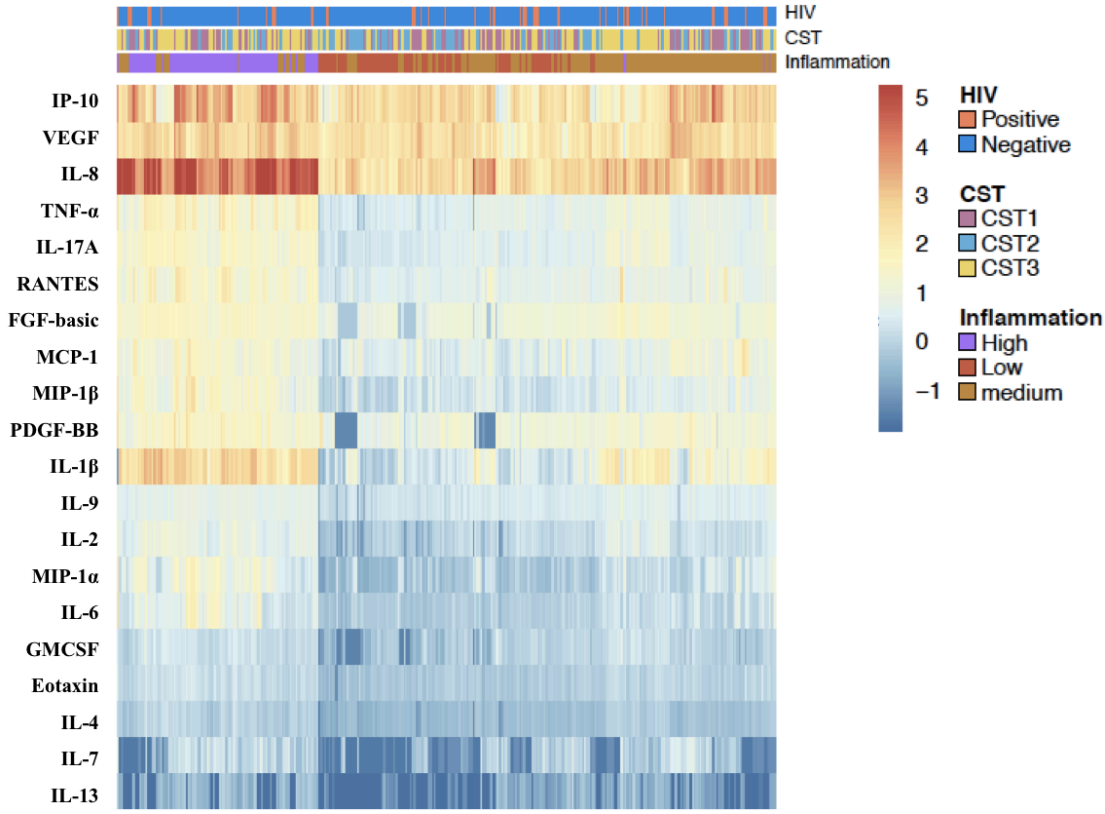


Figure 6. 6 Heatmap showing cytokine distribution by HIV status, community state type and inflammation using supervised hierarchical clustering (Bray Curtis distances) of log_2 transformed standardized counts of cytokines. Each column represents a woman and rows represent individual cytokines. Red colour represents higher concentrations while blue presents lower concentrations of cytokines.

6.2 Discussion

While many previous studies have evaluated the role of vaginal microbiota in transmission of HIV infection in women [9, 167, 168], few have analysed both vaginal microbiota and cytokines in pregnant HIV-infected women. Here, we describe vaginal microbial communities and concentrations of cytokines in HIV-infected versus HIV-uninfected pregnant women.

As expected it was observed in our study that BV and Nugent score were high in pregnant women who are HIV-infected as is expected, Consistently, our study results demonstrated significantly higher alpha and beta diversity in women who were HIV-infected as compared to HIV-uninfected women, consistent with the findings of studies conducted by Buve *et al.,* (2014) and Chehoud *et al.,* (2017). It follows that significant differences in CSTs by HIV status were also observed, with more HIV-infected women having CST3 vaginal microbiota. In contrast, Chehoud and colleagues (2017), in a longitudinal study of non-pregnant women from Chicago, did not find any significant differences in community structure associated with HIV status (p=0.4) among Chicagoan women, and the lack of significant differences was attributed to effective antiretroviral therapy [152].

Our results demonstrated that the relative abundance of particular bacteria also differed by HIV status. *L. crispatus* species were dominant in HIV-uninfected women while there was a reduction in HIV-infected women. Additionally, as expected, a notably high number of BV-associated organisms were significantly more abundant in HIV-infected compared to uninfected women according to metagenomSeq analysis (*G. vaginalis, Megasphaera, P. timonensis, BVAB1, A. vaginae, Sneathia, Mycoplasma, Gemella, P. pallens, Aerococcus, Dialister*). This concurs with previous studies that have reported that dysbiotic vaginal microbiota and BV+ status were associated with HIV acquisition in non-pregnant African women [131, 152, 169, 171, 442]. However, relatively little has been published on HIV and the microbiota in pregnancy in African women.

There is lack of conclusive reproducibility in individual taxa associated with HIV infection in different studies that have been conducted. In some studies performed in non-pregnant women, *Propionibacterineae, Anaerococcus*, *Citrobacter* [172], *G. vaginalis*, *M. hominis* [171], *Prevotella bivia* and *A. vaginae* [145] were associated with HIV status. A study done in pregnant women found that *Mycoplasma* species (*M.*

genitalium, U. urealyticum, M. hominis) were highly associated with HIV infected status [278]. Similar to these previous studies, in our study we found that *Mycoplasma, G. vaginalis*, *Prevotella* species and *A. vaginae* were associated with HIV infection. The inverse relationship between *L. crispatus* and HIV positivity that was demonstrated in our study was also reported by Schellenberg *et al.,* (2011) in a study of non-pregnant sex workers living in Nairobi, Kenya, though their results were statistically insignificant [443]. The variation in findings of various cohort studies may be due to the fact that not all populations have the same dominant microbiota in their vaginal canal as the microbiota is influenced by different physiological and environmental factors

In non-pregnant women, vaginal microbiota modulates genital inflammation; organisms such as *L. crispatus* are associated with reduced inflammation and HIV transmission risk, while vaginal anaerobes such as *Prevotella* and *Sneathia* are associated with increased vaginal inflammation [134, 167]. The presence of high inflammation leads to functional imbalances that affect mucosal surface permeability leading to detrimental health effects [444]. This disruption of mucosal surface permeability during dysbiosis could lead to increased viral shedding, which may impose a higher risk of HIV transmission from pregnant mothers to their partners or infants. In support, it has been shown previously that higher levels of vaginal inflammation were associated with increased risk of HIV shedding [164]. It has also been found that inflammatory cytokines (IL-6, IL-8, IL-1β) were upregulated in cervico-vaginal fluid of acutely HIV infected women with low CD4+ count [445], suggesting an association between mucosal inflammation and degree of immunocompromise [445, 446]. This may partially explain the observation in this study where HIV infected women were more likely to have preterm deliveries as compared to HIV uninfected women since more HIV infected women had high and medium level of inflammation.

However, although in our study, we found high microbial diversity in these HIV-infected pregnant women, few differences in inflammation were observed between HIV-infected and uninfected women were observed. Similarly, in non-pregnant women, another study found that cervicovaginal inflammatory cytokine profiles did not differ when comparing the same women pre- and post- HIV infection [164]. It is possible that the lack of inflammation in our HIV-infected women was due to the fact

that most women were on ART. Maharaj and colleagues (2017) found that HIV-infected pregnant women on HAART had low levels of serum proinflammatory cytokines [437]. This was true in non-pregnant HIV infected women found concurring results in plasma levels of circulating inflammatory biomarkers [439]. This led to the conclusion that the use of HAART suppresses the proinflammatory response, thereby leading to consistently lower cytokine concentrations, although immune activation does not necessarily return to baseline but remains elevated [437, 439, 447]. Given that 95% of HIV-infected women in our cohort were already on HAART treatment long before they became pregnant, with a mean CD4+ count of 532 cell/mm^3 and high degree of viral suppression, this may explain the low levels of inflammation in the genital tracts of HIV-infected compared to uninfected women. Another potential explanation for the lack of inflammation associated with HIV infection in our cohort is that these women were pregnant. Pregnancy is a state of relative immunosuppression characterised by anti-inflammatory responses which are important for embryonic implantation and pregnancy maintenance [448].

In our study, low levels of the Th2 cytokine IL-13, which is important in pregnancy tolerance, was associated with being HIV infected. Previous studies have highlighted the importance of Th2 cytokines in pregnancy, suggesting that imbalances may cause poor pregnancy outcomes [334, 405, 407]. However, in our study there were no significant differences in poor outcomes between HIV-infected and HIV-uninfected women, although there was a notable trend of HIV-infected women being more likely to deliver preterm as compared to HIV-uninfected women. High levels of vaginal TNF-α were independently associated with HIV infection in our study in agreement with a previous study that have reported increased levels of placental TNF-α in HIV-infected women during pregnancy [435] and in vaginal fluid of HIV-infected non-pregnant women compared to HIV-uninfected women [449]. Plasma TNF-α is usually increased in early pregnancy as it is critical for processes of gamete, follicle, placental differentiation and development and parturition among others [321]. However, elevation of TNF-α in the third trimester may be associated with poor pregnancy outcomes such as PTB, LBW or PROM [450, 451]. In HIV infection among other possible reasons, TNF-α is associated with viral control as it engages in selective destruction of virally infected cell [452], thus it is known for increasing TNF- α expression [436], this may explain the high level observed in HIV positive as compared

to HIV uninfected women and a trend of higher preterm delivery in HIV-infected women.

In summary, we affirm previous findings of a positive association of microbial diversity and HIV status, where HIV-infected women had highly diverse vaginal communities as compared to HIV-uninfected women during pregnancy. Despite having highly diverse microbial community, it is interesting to note that HIV-infected women did not have significantly higher levels of genital inflammation overall. The key pro-inflammatory cytokine TNF-α was increased in HIV-infected women, although without any observed association with poor outcomes. Other physiological and chemical factors not considered in this study may need to be studied to expand knowledge on the relationship between HIV, cytokines and microbiota in pregnancy.

Chapter 7 Discussion

The main aim of this thesis was to explore the relationship between vaginal microbiota, BV, HIV and pregnancy outcome in Zimbabwean women. In order to address our main research question, we first characterized the vaginal microbiota of these Zimbabwean pregnant women. We noted that as opposed to studies conducted in North America [51, 58],pregnant Zimbabwean women had a high prevalence of dysbiosis and *L. iners* was the most common organism identified. The vaginas of these pregnant women were predominantly colonised by a milieu of diverse anaerobic bacteria similar to that found in non-pregnant sub Saharan African women and women of African descent [51, 58, 135, 145]. The widely expressed view that an optimal vaginal environment is *Lactobacillus* spp dominant [57] may not be true for all populations, as it is consistently evident that the optimal vaginal microbiota for black women is dysbiotic [85, 145]. There has been an appreciation of the differences of the vaginal microbiota according to ethnic and geographical background from recent studies [51, 57, 58]. Our study adds more data that sub Saharan African pregnant women also have a tendency toward dysbiosis. We also confirmed the prevalence of *L. iners* in the vaginas of women in this cohort and its ubiquitous nature of surviving even in dysbiotic environment unlike other *Lactobacillus* spp [57, 67, 453].

Next, we looked at vaginal microbiota and pregnancy outcomes. Although there were weak associations between preterm birth and vaginal microbiota, there was an association between vaginal microbiota and low birth weight and SGA. Surprisingly, women with BV were less likely to deliver LBW infants and there was a significantly lower alpha diversity in the vaginas of women who delivered LBW infants. On further probing, the relative abundance of *L. iners* was most abundant in women who delivered LBW infants therefore explaining the association with lack of BV and low diversity. Since *L. iners* was the most prevalent *lactobacillus* in this cohort and in other African women, this may partly explain why the rates of LBW are so high in this setting.

G. vaginalis and *A. vaginae* were seemingly lower in pregnancies that resulted in LBW neonates in this particular cohort using various methods. This differs from the existing dogma that an unhealthy vaginal microbiota is a cause of adverse birth outcomes. However, it may be that their relative abundance was lower as a result of the increased relative abundance of *L. iners.* This speculation could be validated using quantitative PCR. None-the-less, our findings suggest that a low abundance of *G. vaginalis* and *A.*

vaginae seem to correlate adverse birth outcomes. This is possibly due to their inflammatory nature and the fact that lack of inflammation in the vagina was associated with good pregnancy outcomes.

The prevalence of bacterial vaginosis in these pregnant women was high (32%) similar to data from previously published Zimbabwean cohorts [28]. While we did not observe any association between BV and PTB or SGA, lack of BV was associated with LBW. This however contrast the existing dogma that a dysbiotic vaginal environment is associated with poor birth outcomes. This baseline information is important in this particular population, however, there is need to validate these results in a longitudinal cohort in pregnant women. Furthermore, it is imperative to also establish the vaginal microbiota of women in this population in non-pregnant women and adolescents. This is important as these findings would be linked with possible research on interventions whenever needed for this population.

We hypothesized that there would be differences in the vaginal microbiota in pregnant HIV infected and HIV uninfected women. We were able to confirm our hypothesis, as we found that HIV-infected women had significantly higher alpha diversity and their microbial communities clustered significantly differently to that of HIV negative women, despite the fact that most, if not all, of the cohort were on combination ART. We were able to establish the strong relationship between HIV and CST3. Some individual taxa (*Megaspahaera* and *Mycoplasma*) were highly associated with HIV infection, similar to that in other pregnant [278, 454] and non-pregnant cohorts [219]. Knowledge of organisms associated with HIV status is of paramount importance for health management since our population has a high prevalence and incidence of HIV.

We analysed the immunological biomarkers and possible predictors of bacterial vaginosis and adverse pregnancy outcomes by measuring vaginal cytokine concentrations. We observed that *L. iners* (CST1) and *G. vaginalis* (CST3) dominated CSTs were highly inflammatory with high levels of IL-1β, TNFα, IL-8 and IL-6 as compared to CST2 (*L. crispatus* dominant). Similarly, individual taxa such as *G. vaginalis, Dialister, A. vaginae, Aerococcus, Prevotella*, *L. iners, Megasphaera* and*BVAB1* were associated with high inflammation while *L. crispatus* was associated with low inflammation in line with many previous studies [46, 411, 412]. And as expected inflammation was higher in BV+ women as compared to BV-.

Low levels of Th2 cytokine (IL-13) were highly associated with LBW, PTB, SGA. Additionally, low levels of growth factor PDGF-BB were associated with PTB and LBW while the associations were not upheld in SGA delivery after multiple comparisons. Growth factors act as placental growth regulators and play an important role in fetal development [455]. Th2 cytokines are thought to be important for pregnancy tolerance [340]. Therefore, low levels of theses cytokines this might explain the reason for the association with poor birth outcomes. However, due to the nature of our study design, results are inconclusive since our study was cross sectional. However, longitudinal studies would provide substantiated evidence when many time points are evaluated throughout pregnancy.

As expected, pregnancy induced hypertension was a risk factors associated with PTB, LBW and SGA in line with a previous study done in Zimbabwe [401]. We think this is unlikely to be solely due to induction of labour, since this is uncommon practice in Zimbabwe unless in case of severe complications. HIV infection was a risk factor associated with PTB and LBW, while GBS carriage was a risk factor for SGA deliveries. Furthermore, a history of previous poor outcomes was associated with LBW delivery. There were more HIV infected women who experienced poor birth outcomes as compared to HIV uninfected women in this high-risk cohort in line with findings from previous studies [192, 401]. The reason for this finding is difficult to ascertain as poor birth outcome causes are multifactorial. Although some have reported poor birth outcomes associated with ART use [22, 188], others have reported the contrary [194-196] HIV infection was associated with and low levels of IL-13, and IL-7 cytokines which are important for angiogenesis, implantation and development of the foetus. While on the other hand HIV was associated with high levels of TNF-α which has been reported to be detrimental in third trimester as compared to first trimester, However, we did not we did not find any associations between HIV and poor birth outcomes.

Finally, its satisfying to know that this study has added to the base of the scientific knowledge on vaginal microbiota of sub Saharan African women during pregnancy and created a basis for further studies and research in this area. Specifically, this thesis has provided valuable insight on relationship between BV, vaginal microbiota and pregnancy outcomes an area that had not been explored in the Zimbabwean population.

7.1 Limitations

- We should stress that our study was limited by the kind of study design employed. Cross sectional microbiome studies tend to be variable and difficult to reproduce. Therefore, due to the fact that samples were collected at a single time-point in this study, future longitudinal studies would be important to confirm these findings. This will give more reliable results which may show possible variation at different stages of pregnancy.
- 16S rRNA amplicon sequencing does not provide information about non-bacterial microbial taxa that also constitute the vaginal microbiome and some bacteria are only classified to genus and not species level therefore using our data for inferences should be treated with caution.
- Microbiota and cytokines were assessed in samples collected from women at different stages in their pregnancies and this could have introduced heterogeneity that may mask certain gestational age-associated relationships.
- The study was underpowered to truly assess differences by PTB status and HIV status as there were relatively low numbers of both.
- Our study was limited in determining accurate gestational age of the pregnancies. Very few women had access to ultrasound scans, therefore manual methods such as last menstrual period and fundal height were used which might lead to inaccurate PTB classification.
- The fact that our cohort was composed of a mostly high-risk referral cases might limit the generalizability our findings.
- The failure to pass QC by other cytokines such as IL-12p70, IL-15, IL-10, IL-5, GCSF and IFN-γ has greatly affected our analysis. We were not able to make reliable analysis since some important Th1 and Th2 cytokines we were interested in during our protocol design plan.
- Data on recent sexual activity, which can impact the vaginal microbiota and may differ in pregnancy, was not collected at enrollment.

7.2 Future studies

Longitudinal studies should be pursued to give a comprehensive conclusion to the relationships between vaginal microbiota at all trimesters in relation to pregnancy outcomes in a non- risky cohort. Furthermore, there is need to employ whole genome sequencing technique which would give information as to the function of the microbiota

and would help to characterise some organisms to species level which we were not able to characterised fully using the V4 hypervariable region. Future studies can also be conducted in pregnant women from both urban and rural areas and also include non-pregnant women of reproductive age and adolescents to understand the population vaginal microbiota.

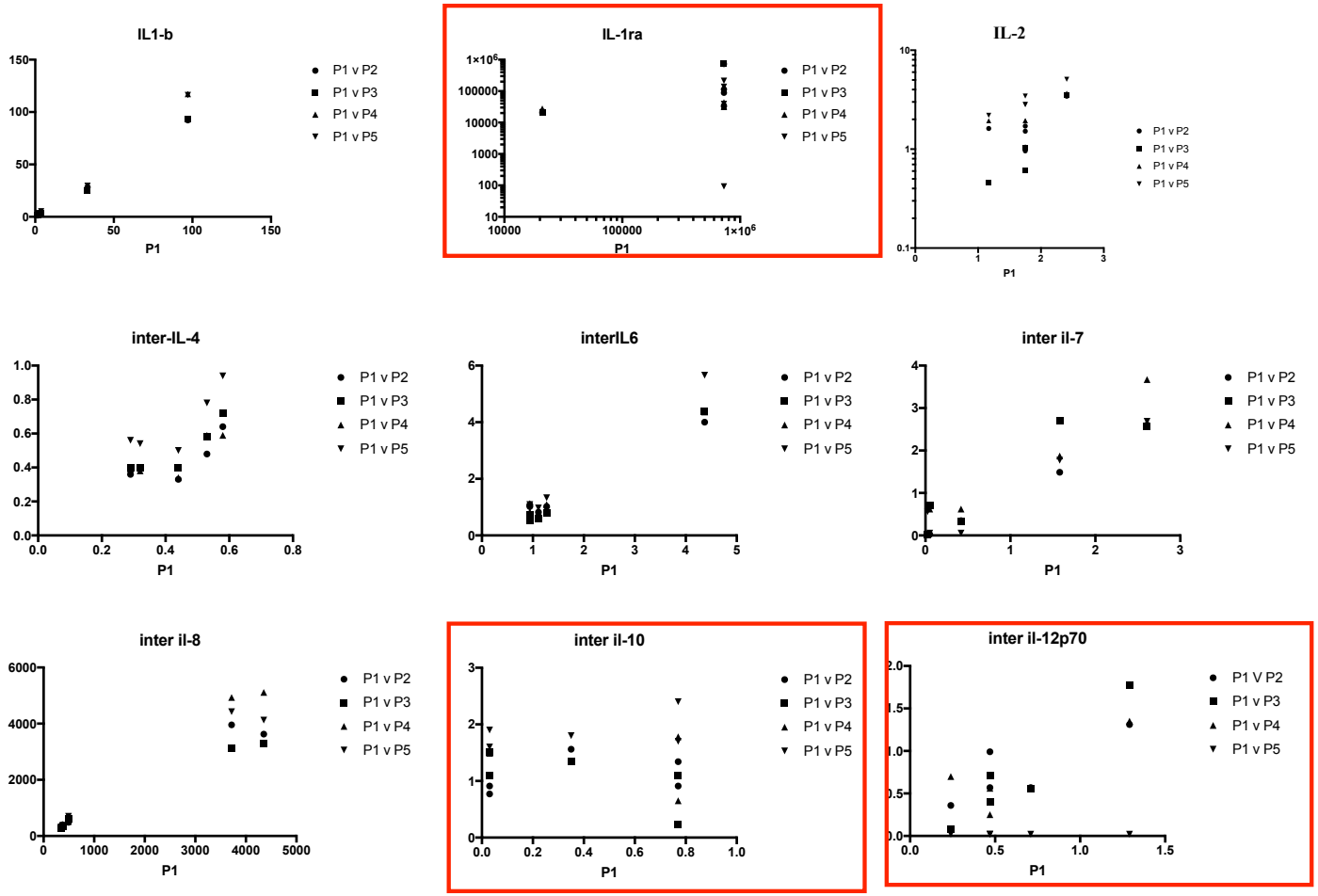
References

Appendix A: Cytokine analysis Interplate controls

	Plate 1 vs Plate 2	Plate 1 vs Plate 3	Plate 1 vs Plate 4	Plate 1 vs Plate 5	Plate 2 vs Plate 3	Plate 2 vs Plate 4	Plate 2 vs Plate 5	Plate 3 vs Plate 4	Plate 3 vs Plate 5	Plate 4 vs Plate 5
IL-1b	1.00	0.90	1.00	0.90	0.90	1.00	0.90	0.90	1.00	0.90
IL-1ra	0.71	1.00	0.71	0.35	0.71	0.70	0.60	0.70	0.35	0.90
IL-2	0.45	0.92	0.50	0.91	0.62	0.89	0.67	0.69	0.92	0.57
IL-4	0.70	0.89	0.56	0.60	0.89	0.87	0.90	0.86	0.89	0.97
IL-5	0.24	0.53	0.24	0.29	0.79	-0.05	-0.11	0.53	0.50	0.95
IL-6	0.29	0.82	0.50	0.76	0.67	0.92	0.76	0.87	0.97	0.89
IL-7	0.97	0.80	0.97	0.67	0.72	0.92	0.76	0.87	0.56	0.68
IL-8	0.90	1.00	1.00	0.90	0.90	0.90	1.00	1.00	0.90	0.90
IL-9	0.87	0.97	0.97	0.97	0.90	0.90	0.80	1.00	0.90	0.90
IL-10	0.49	-0.65	-0.16	0.32	-0.13	-0.03	0.62	0.29	-0.36	0.56
IL-12(p70)	0.76	0.82	0.29	0.00	0.97	0.29	0.00	0.36	0.00	0.00
IL-13	0.95	0.87	0.53	0.89	0.82	0.55	0.97	0.67	0.74	0.65
IL-15	0.87	0.50	-0.21	-0.05	0.67	0.11	0.16	0.74	0.05	0.16
IL-17	0.90	0.90	0.70	0.70	1.00	0.90	0.90	0.90	0.90	1.00
Eotaxin	0.60	0.90	0.70	0.60	0.80	0.90	1.00	0.90	0.80	0.90
FGF basic	0.53	0.50	0.34	0.50	0.92	0.92	0.92	0.76	1.00	0.76
G-CSF	0.67	0.15	0.87	0.87	0.00	0.60	0.50	0.60	0.50	0.90
GM-CSF	0.90	0.80	0.30	0.70	0.90	0.60	0.90	0.70	0.70	0.70
IFN-g	-0.10	0.50	0.10	0.30	0.70	0.80	0.90	0.80	0.90	0.90
IP-10	1.00	0.90	0.90	0.90	0.90	0.90	0.90	1.00	1.00	1.00
MCP-1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MIP-1a	0.70	1.00	0.97	1.00	0.70	0.82	0.70	0.97	1.00	0.97
PDGF-bb	0.29	0.29	0.76	0.97	0.76	0.71	0.41	0.68	0.36	0.87

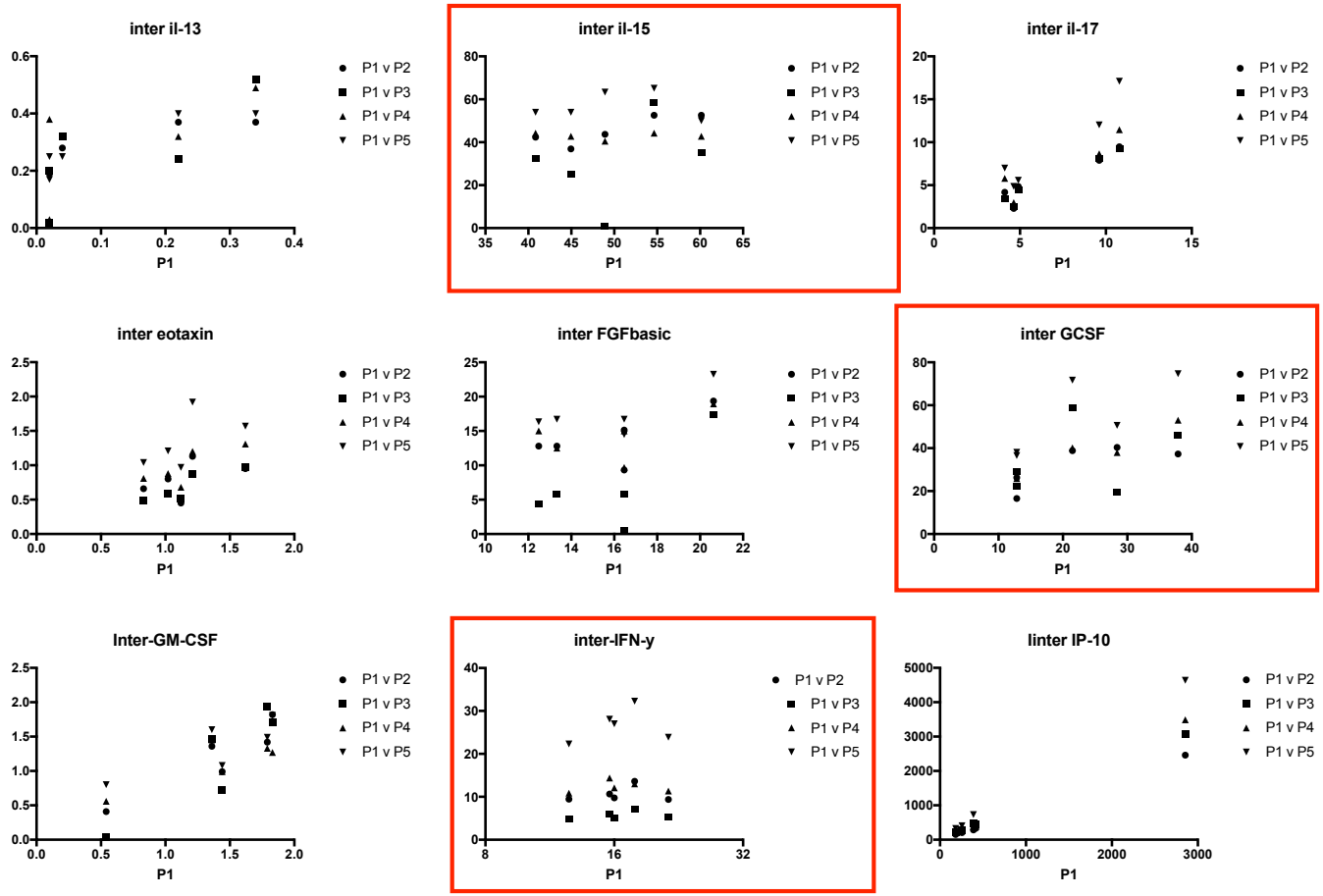
MIP-1b	0.90	0.90	1.00	1.00	1.00	0.90	0.90	0.90	0.90	1.00
RANTES	1.00	0.90	0.90	0.90	0.90	0.90	0.90	1.00	1.00	1.00
TNF-a	0.82	0.97	0.97	1.00	0.90	0.90	0.82	1.00	0.97	0.97
VEGF	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Appendix B1 Cytokine interplate control



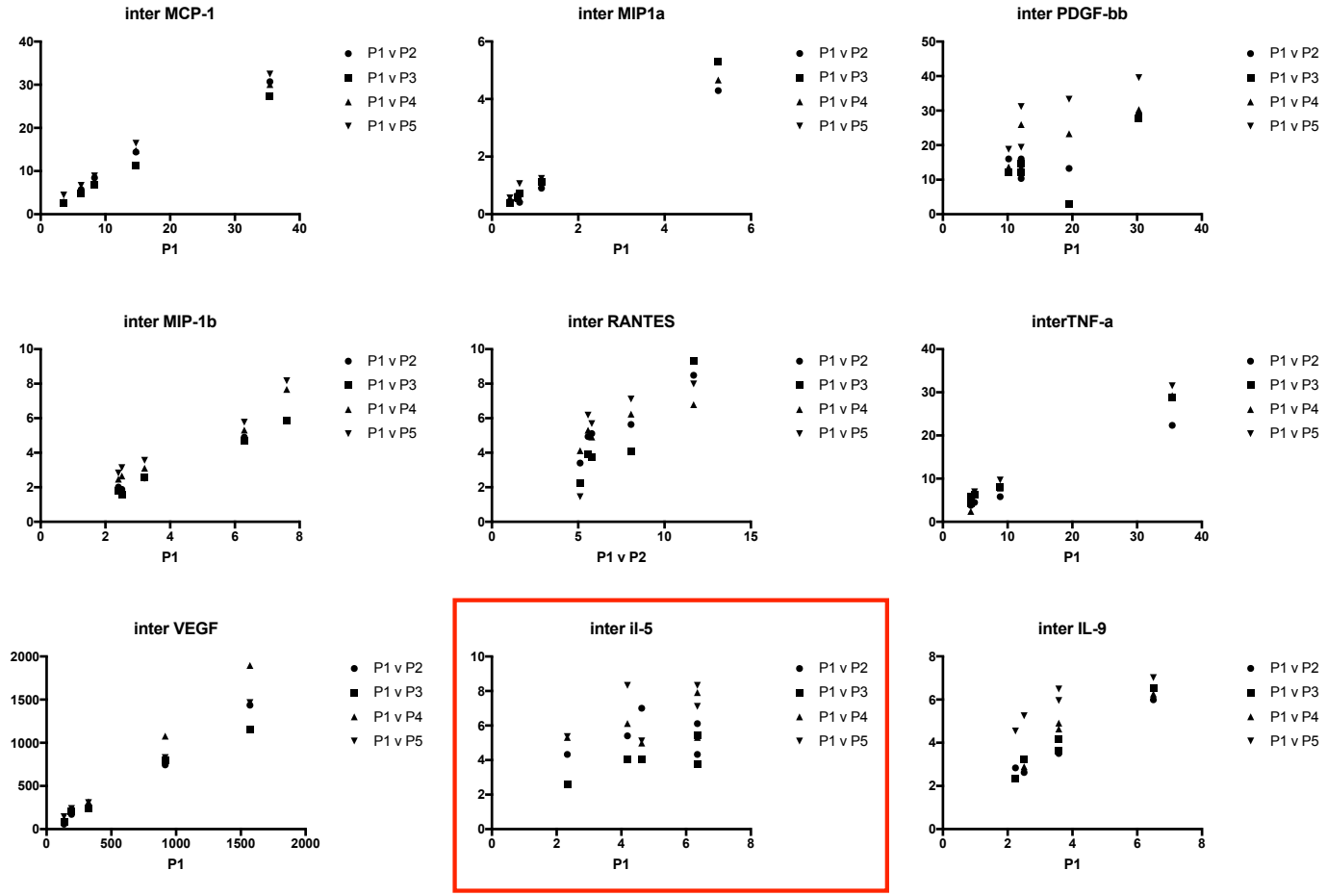
The cytokines highlighted in red boxes failed the interplate quality control and were excluded from the analysis

Appendix B2 Cytokine interplate controls



The cytokines highlighted in red boxes failed the interplate quality control and were excluded from the analysis

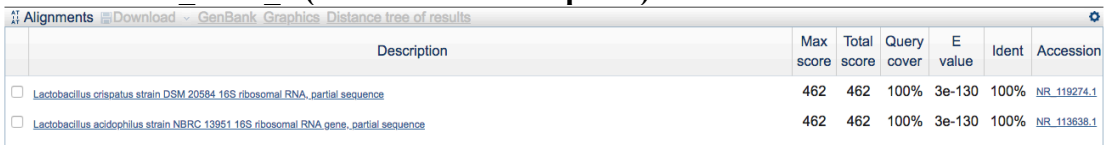
Appendix B3 Cytokine interplate controls



The cytokines highlighted in red boxes failed the interplate quality control and were excluded from the analysis

Appendix C: NCBI Blasting Annotations

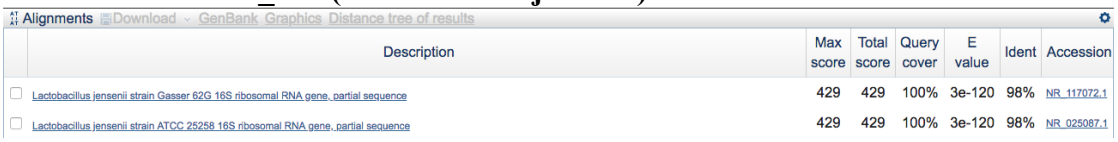
Lactobacillus_OUT_9 (Lactobacillus Crispatus)



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lactobacillus crispatus strain DSM 20584 16S ribosomal RNA, partial sequence	462	462	100%	3e-130	100%	NR_119274.1
Lactobacillus acidophilus strain NBRC 13951 16S ribosomal RNA gene, partial sequence	462	462	100%	3e-130	100%	NR_113638.1

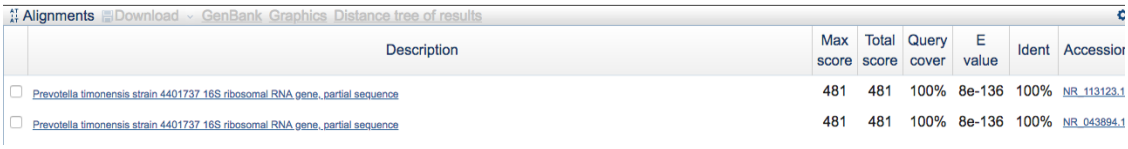
Lactobacillus OUT_745 (Lactobacillus jensenii)



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lactobacillus jensenii strain Gasser 62G 16S ribosomal RNA gene, partial sequence	429	429	100%	3e-120	98%	NR_117072.1
Lactobacillus jensenii strain ATCC 25258 16S ribosomal RNA gene, partial sequence	429	429	100%	3e-120	98%	NR_025087.1

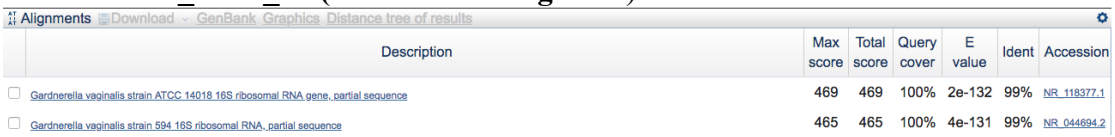
Prevotella OUT_8 (Prevotella timonensis)



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accessio
Prevotella timonensis strain 4401737 16S ribosomal RNA gene, partial sequence	481	481	100%	8e-136	100%	NR_113123.1
Prevotella timonensis strain 4401737 16S ribosomal RNA gene, partial sequence	481	481	100%	8e-136	100%	NR_043894.1

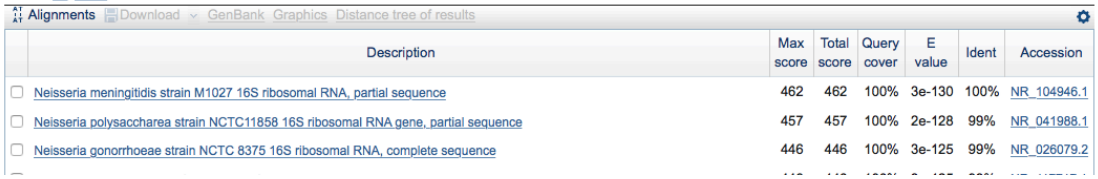
Gardnerella _OTU_11 (Gardnerella vaginalis)



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Gardnerella vaginalis strain ATCC 14018 16S ribosomal RNA gene, partial sequence	469	469	100%	2e-132	99%	NR_118377.1
Gardnerella vaginalis strain 594 16S ribosomal RNA, partial sequence	465	465	100%	4e-131	99%	NR_044694.2

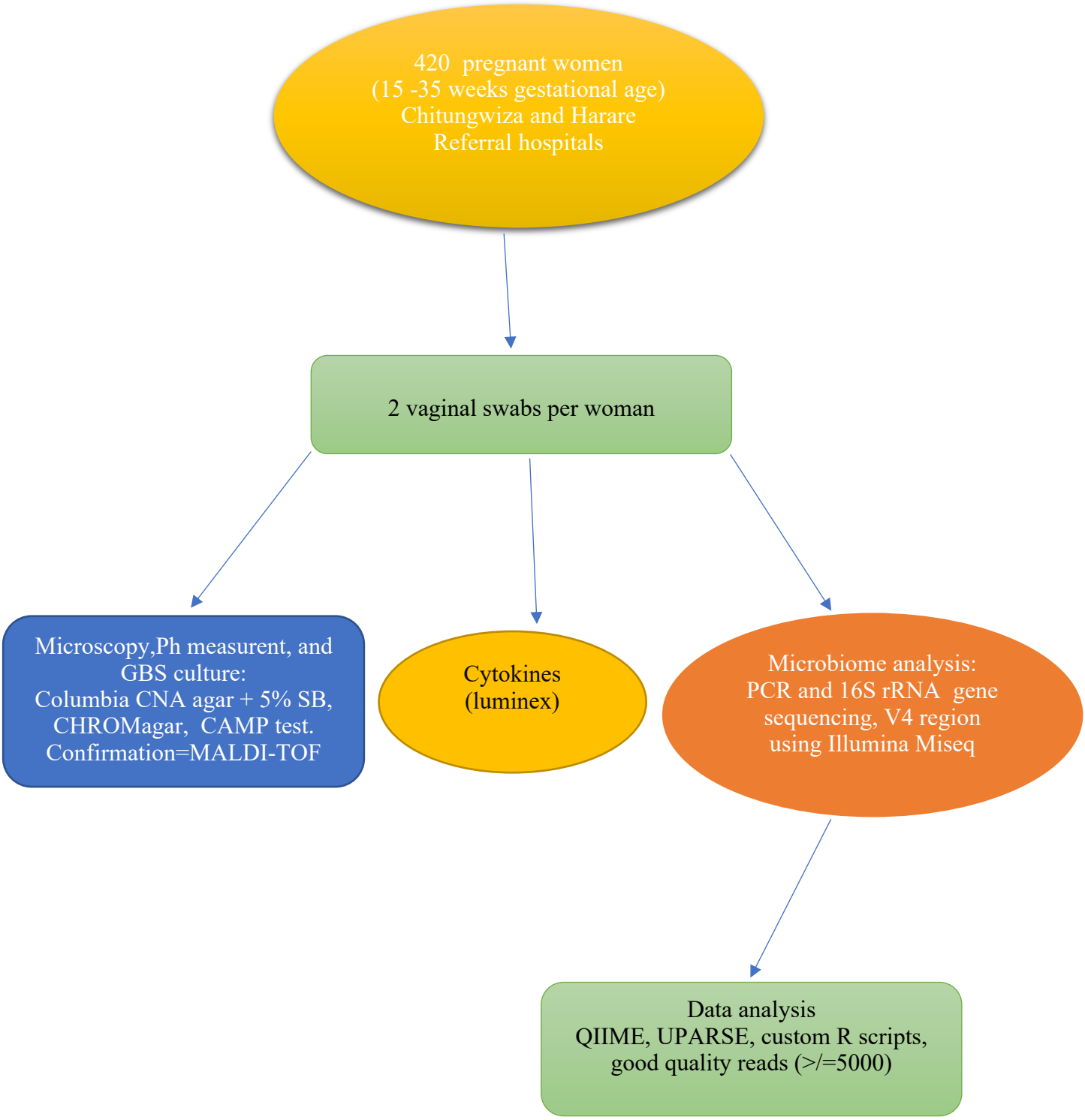
Neisseria _OTU_40 (Neisseria gonorrhoeae)



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Neisseria meningitidis strain M1027 16S ribosomal RNA, partial sequence	462	462	100%	3e-130	100%	NR_104946.1
Neisseria polysaccharea strain NCTC11858 16S ribosomal RNA gene, partial sequence	457	457	100%	2e-128	99%	NR_041988.1
Neisseria gonorrhoeae strain NCTC 8375 16S ribosomal RNA, complete sequence	446	446	100%	3e-125	99%	NR_026079.2

Appendix D: Methodology summary



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